NEUROENDOCRINE MECHANISMS OF BEHAVIORAL CHANGES INDUCED BY HYPOGLYCEMIA

BY

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DISSERTATION

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ABSTRACT

Hypoglycemia is associated with a variety of adverse behaviors including fatigue, confusion, social withdrawal, anhedonia and depressive-like behaviors. While these clinical symptoms are well characterized, the mechanisms of their cause are not understood. Here, we investigated how insulin-induced hypoglycemia causes social withdrawal and changes in mood. To investigate effects on social withdrawal, male 8-12-week-old C57BL/6J mice were injected intraperitoneally with saline and/or insulin (0.8 or 1.2units/kg). Insulin generated significant hypoglycemia with the lowest blood glucose levels of 64±4 and 48±5mg/dl for 0.8 and 1.2units/kg of insulin, respectively. Insulin at either dose caused near total social withdrawal at 0.75h, with full recovery not occurring until 4h (0.8units/kg) or 8h (1.2units/kg) post-insulin injection. Insulin also caused a marked elevation in plasma catecholamines. Basal 12h fasting norepinephrine (NE) and epinephrine (Epi) were 287±38 and 350±47pg/ml, respectively. Insulin at 0.8units/kg increased plasma NE and Epi to 994±73 and 1842±472 pg/ml, respectively. Administration of exogenous NE or Epi caused social withdrawal similar in magnitude to insulin. Importantly, administration of the β-2 adrenergic receptor agonist terbutaline also caused social withdrawal, while administration of the β-2 adrenergic receptor antagonist butoxamine blocked NE-induced social withdrawal. Finally, butoxamine blocked insulin-induced social withdrawal.

These data demonstrate that hypoglycemia-associated social withdrawal is dependent on catecholamines via a β-2 receptor-mediated pathway.

Next, we investigated how insulin-induced hypoglycemia causes anhedonia and depressive-like behavior. Saccharin preference testing 24h post hypoglycemia showed that mice receiving insulin (0.8 units/kg) had saccharin aversion (62% vs 90.5% of total fluid consumption). In addition, mice administered insulin had increased immobility in the forced swim test that took 48h to rectify. Insulin at 0.8units/kg increased plasma corticosterone (325±23pg/ml vs. 119±32pg/ml), Epi (814±254pg/ml vs. 350±40pg/ml), and NE (541±155pg/ml vs. 265±28pg/ml) at 24h post insulin treatment. Importantly, blocking of the adrenergic receptors with phentolamine, metoprolol and butoxamine, or treatment with the anti-depressants (fluoxetine and desipramine) ablated the insulin-induced saccharin aversion and increased immobility in forced swim test. Taken together, these data indicate that anhedonia and depressive-like behaviors are induced by hypoglycemia and those behaviors are dependent on catecholamines in an adrenergic receptor-mediated manner.

Dedicated to those who inspired me, my family

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Ach	Acetylcholine
ACTH	Adrenocorticotropic hormone
AMPK	AMP-activated protein kinase
AR	Adrenergic Receptor
BTX	Butoxamine
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
Epi	Epinephrine
FST	Forced swim test
gp 130	Glycoprotein 130
HPA axis	Hypothalamic–pituitary–adrenal axis
hsCRP	High-sensitivity C-reactive protein
IFN-γ	Interferon-gamma
ICU	Intensive care units
ICV	Intracerebroventricular
IGF	Insulin-like growth factor
IGT	Impaired glucose tolerance
IIT	Intensive insulin therapy
IL	Interleukin
IL-1RA	Interleukin 1 receptor antagonist
IL-1R2	Interleukin 1 receptor, type II
Ins	Insulin
IP	intraperitoneal
IRS	Insulin receptor substrate
JAK-2	Janus kinase-2
LC	Locus coeruleus
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
Meto	Metoprolol
mTOR	Mammalian target of rapamycin
MYD88	Myeloid differentiation primary response gene (88)
NE	Norepinephrine
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

PET	Positron emission tomography
Phen	Phentolamine
PI3K	Phosphotidylinositide 3’-kinase
PPARγ	Peroxisome proliferator-activated receptor gamma
SH-2	Src Homology 2
SOCS	Suppressor of cytokine signaling
SPT	Saccharin preference test
SSI-1	STAT-induced STAT inhibitor
STAT-3	Signal transducer and activator of transcription-3
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
Terb	Terbutaline
TNF-α	Tumor necrosis factor-alpha
TST	Tail suspension test

CHAPTER 1: INTRODUCTION

1.1 Significance

Hypoglycemia is the most common side effect of drugs used to treat diabetes. In humans, the normal range of fasting plasma glucose concentration is approximately 70-110 mg/dl (3.9-6.1 mmol/L) and it is maintained within a narrow range. Glucose levels < 55 mg/dl (3.0 mmol/L) with symptoms that are relieved promptly after the glucose level is raised document hypoglycemia (Braunwald, 2001). People with Type 1 Diabetes (T1D) suffer an average of two episodes of symptomatic hypoglycemia per week. An estimated 2~4 % of people with T1D die as a result of hypoglycemia each year. Hypoglycemia occurs to a similar degree in Type 2 Diabetes (T2D), as with T1D, because both require insulin treatment and develop insulin deficiency. Thus, hypoglycemia is called a “fact of life” for people with T1D and T2D (Braunwald, 2001).

Symptoms of hypoglycemia may be adrenergic in origin due to epinephrine (Epi) release or related to neuroglycopenia (Hoffman et al., 1997; Hoffman, 2007; Korytkowski et al., 1998; Service, 1995; Ste Marie and Palmiter, 2003). The adrenergic symptoms include: tremor, pallor, rapid heart rate, palpitations and diaphoresis (Binder and Bendtson, 1992; Bolli, 1997; Korytkowski et al., 1998). Neuroglycopenic symptoms

range from fatigue, lethargy, headache, drowsiness and behavior change to seizures, unconsciousness and coma (Binder and Bendtson, 1992).

In addition to those physical symptoms, psychological symptoms are reported. In a study of hypoglycemic patients, Gyland (1953) reported several symptoms of depression occurring in 77% of the patients and worrying and anxiety in 62% of the patients (Gyland, 1953). More recently, positron emission tomography (PET) scans have verified that glucose metabolism is often reduced in the brains of patients suffering from depression (Kennedy et al., 2001).

Although hypoglycemia and its component syndromes are well-characterized clinically as explained above, the mechanisms underlying these conditions remain largely unknown (Cryer, 2004). Herein, we investigated the mechanism underlying hypoglycemia-induced sickness behavior and depression. The main objectives of this proposal were to: 1) investigate how insulin-induced hypoglycemia causes social withdrawal, and 2) determine how hypoglycemia causes depressive-like behavior and anhedonia.

The scope of this research proposal was to study the impact of hypoglycemia on the physiological and psychological symptoms. It is important to the field of psychoneuroimmunology to determine the mechanisms by which low blood glucose

influences activation of the adrenergic system and subsequent biobehaviors and mood disorders. This project is rooted in the inherent idea as well as scientific evidence suggesting that low blood sugar affects the adrenergic system, more specifically, endogenous NE and Epi that are known mediators of social withdrawal and depression.

1.2 Objectives

The objective of this work was to characterize the possible mechanisms of behavior and mood changes induced by hypoglycemia. The hypothesis of this dissertation is that insulin-induced hypoglycemia causes social withdrawal and depressive-like behaviors mediated by neuroendocrine mechanisms.

Therefore, the first objective of this dissertation was to determine how insulin-induced hypoglycemia causes social withdrawal. This included the following questions: 1) is insulin-induced hypoglycemia associated with social withdrawal?; 2) do catecholamines cause social withdrawal?; 3) does β-2 adrenergic receptor stimulation cause social withdrawal?; and 4) does β-2 adrenergic receptor antagonism prevent insulin-induced social withdrawal?

The second objective of this dissertation was to investigate how insulin-induced hypoglycemia causes depressive-like behavior and anhedonia. To answer this question,

we were interested in determining if insulin-induced hypoglycemia associated with depressive-like behavior and anhedonia. The subsequent question then became: what is the mechanism and how can we possibly reverse the adverse behaviors? Further studies were conducted to answer: 1) does an increase in catecholamines mediate depressive-like behavior in insulin-induced hypoglycemia? ?; 2) do exogenous catecholamines cause depressive-like behavior?; 3) do exogenous catecholamines induce anhedonia?; 4) are adrenergic receptors associated with depressive-like behavior and anhedonia?; and 5) does an antidepressant prevent hypoglycemia-induced depressive-like behavior?

CHAPTER 2: LITERATURE REVIEW

2.1 Type 1 Diabetes (T1D)

Interactions of genetic, environmental, and immunologic factors that ultimately lead to the destruction of the pancreatic β cells and insulin deficiency are the underlying cause of T1D. Ultimately, autoimmune β cell destruction occurs and most, but not all, individuals with T1D have evidence of islet-directed autoimmunity. However, some individuals who have the clinical phenotype of T1D lack immunologic markers indicative of an autoimmune process involving the β cells. These individuals are thought to develop insulin deficiency by unknown, non-immune mechanisms (Braunwald, 2001).

Pathologically, the pancreatic islets are infiltrated with lymphocytes, resulting in insulitis. After all β cells are destroyed, the inflammatory process abates, the islets become atrophic, and most immunologic markers disappear. B cells seem to be particularly susceptible to the toxic effect of some cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-1 (IL-1) (Goodman et al., 2006). The precise mechanisms of β cell death are not known but may involve formation of nitric oxide metabolites, apoptosis and direct CD8+ T cell cytotoxicity. The islet destruction is mediated by T-lymphocytes, rather than islet autoantibodies, as these

antibodies do not generally react with the cell surface of islet cells and are not capable of transferring diabetes to animals (Goodman et al., 2006).

Numerous environmental events have been proposed to trigger the autoimmune process in genetically susceptible individuals; however, none have been conclusively linked to diabetes. Putative environmental triggers include viruses (coxsackie and rubella most prominently), bovine milk proteins and nitrosourea compounds.

2.2 Type 2 Diabetes (T2D)

Type 2 diabetes affects more than 150 million people worldwide (Matthaei et al., 2000) and is projected to increase to 300 million by the year 2020 (Crook, 2004). Unlike T1D, which is characterized by an absolute lack of insulin, T2D is characterized by defective insulin function which progresses from subclinical impaired glucose intolerance and insulin resistance to overt diabetes over the course of years (Zimmet et al., 2001). Importantly, during this subclinical phase of the disease, health complications such as atherosclerosis and low grade chronic inflammation are already present (Zimmet and Alberti, 1997). Inflammation is classically defined by four symptoms: swelling, redness, pain and heat. In 1941, Menkin conducted a series of simple, but elegant, experiments that established a firm link between diabetes and inflammation. He found

that pancreactomized dogs injected with an irritant into the pleural cavity showed a nearly 85% increase in blood glucose accompanied by proteolysis, enhanced gluconeogenesis and infiltration with vacuolized polymorphonuclear cells. Non-diabetic dogs showed no change in blood glucose and normal leukocytes after injection of the irritant (Menkin, 1941). Importantly, Menkin was able to block this inflammatory reaction by administration of insulin (Crook, 2004). These findings illustrate that inflammation enhances the severity of diabetes and that diabetes enhances inflammation.

Since this initial finding, thousands of studies have improved our understanding of the interaction between diabetes and inflammation. A decade ago, Pickup and colleagues suggested that T2D was a proinflammatory disease involving activation of the innate immune system (Pickup and Crook, 1998; Pickup, 2004). In support of this concept, subjects with T2D often have elevated serum concentration of acute-phase reactants, including sialic acid, α-1 acid glycoprotein, amyloid A, IL-1β, TNF-α, IL-6, C-reactive protein and cortisol (Menkin, 1941; Zimmet and Alberti, 1997).

Additional studies have shown that altered innate immunity and chronic inflammation appeared strongly associated with insulin resistance in obesity. Uysal et al. (1997) reported that the proinflammatory cytokine TNF-α was synthesized by adipocytes and was a mediator of insulin resistance in obesity. Furthermore, Tilg and colleagues

demonstrated that inactivation of IKK-β prevented fat-induced insulin resistance in (Tilg and Moschen, 2008) skeletal muscle, suggesting it as a potential therapeutic target for T2D. In support of this concept, NF-κB activation and proinflammatory cytokine production (including IL-6, IL-1 and TNF-α) were shown to be increased in the liver by obesity and high-fat diet, leading to insulin resistance and hyperglycemia (Cai et al., 2005). A study by Solinas and coworkers (2007) showed that inflammation, but not obesity per se, triggered insulin resistance. In mice, high-fat diet-induced insulin resistance could be prevented through blocking an inflammatory pathway in macrophages by JNK1 deletion (Solinas et al., 2007). These results suggest that obesity-induced inflammation increases high-fat diet-induced insulin resistance as well as resultant T2D.

Recent data have shown that increased levels of inflammatory cytokines, such as IL-6 and high-sensitivity C-reactive protein (hsCRP), were linked to an elevated risk of clinical diabetes (Cai et al., 2005). Work by Solinas's group indicated that the enhanced proinflammatory phenotype in T2D not only affected complications like cardiovascular disease (Solinas et al., 2007) but also exacerbated other pathologies such as depression and social withdrawal induced by activation of the innate immune system with lipopolysaccharide (Liu et al., 2007; Pradhan et al., 2001; Pradhan et al., 2001) or

hypoxia (Pradhan et al., 2001).

In addition to elevation of proinflammatory cytokines, T2D may be associated with a less effective anti-inflammatory response. The process of insulin resistance has been an area of prolific study. There are several factors that can lead to insulin resistance including increased degradation of the receptors by the proteasome, alteration of downstream signaling partners and phosphorylation at inhibitory serine and threonine residues (Pradhan et al., 2003). One of the critical regulators of this process is a class of molecules called suppressor of cytokine signaling (SOCS). Interestingly, several anti-inflammatory cytokines including insulin-like growth factor-1 (IGF-1), IL-4 and IL-10 share key signaling components with the insulin receptor and are susceptible to similar resistance mechanisms.

2.3 The implication of proinflammatory cytokines in type 2 diabetes[1]

Introduction

The incidence of T2D is rapidly expanding. Some of the more obvious

[1] Guest, C. B., Park, M. J., Johnson, D. R., & Freund, G. G. (2008). The implication of proinflammatory cytokines in type 2 diabetes. Frontiers in Bioscience : A Journal and Virtual Library, 13, 5187-5194.

pathologies associated with it include: defective glucose metabolism, obesity, cardiovascular disease and an inability to mount an effective immune response to infection by certain pathogenic organisms, leading to sepsis and death. A common tie linking these seemingly disparate complications is chronic inflammation. Today we know that inflammation is regulated locally and systemically by numerous biochemical signals. One of the most important of these signals is a class of molecules called cytokines. Cytokines can be generally classified as proinflammatory or anti-inflammatory and allow an organism to respond rapidly to an immune challenge by coordinating an appropriate immune response. In T2D, the balance between proinflammatory and anti-inflammatory cytokines is shifted toward proinflammation, potentially causing or exacerbating the health complications found in T2D. Over-nutrition has been shown to trigger the innate immune system but activation of the innate immune system, itself, induces hyperglycemia and insulin resistance. In all likelihood, diabetes and chronic inflammation are inseparable and act as a reciprocal feed-forward loop.

Proinflammatory Cytokines

There are a variety of cytokines labeled as proinflammatory. Almost all immune

cells as well as epithelial cells and adipocytes produce a subset of these cytokines. Generally, proinflammatory cytokines are important for initiating the innate immune response and for directing the subsequent adaptive immune response. The most studied contributors to the chronic inflammation seen in T2D are leptin, TNF-α, IL-1β and IL-6.

Leptin

Leptin was first discovered after a series of parabiosis experiments (Coleman, 1973). Coleman infused the plasma of obese, hyper-leptinemic *db/db* mice into wild-type mice. Surprisingly, the mice became anorexic and died of starvation. Zhang *et al.* cloned the gene encoding the 16 kDa leptin protein (Zhang et al., 1994), while the gene encoding the principal leptin receptor was identified by Leiter *et al.*(Leiter et al., 1980). The crystal structure of leptin revealed a four-helix bundle similar to that of IL-6 (Zhang et al., 1997). The action of leptin is primarily mediated through Janus kinase-2 (JAK-2) and signal transducer and activator of transcription-3 (STAT-3). Targeted disruption of STAT-3 in the central nervous system induces a phenotype similar to mice lacking either leptin or the leptin receptor (*i.e*. obesity, diabetes and infertility) (Gao et al., 2004). Importantly, leptin has also been shown to act on pathways that include those containing insulin receptor substrate (IRS), phosphotidylinositide 3'-kinase (PI3K), mitogen

activated protein kinase (MAPK) (Martin-Romero and Sanchez-Margalet, 2001) and, recently, AMP-activated protein kinase (Minokoshi et al., 2002) (for a complete review see (Fruhbeck, 2006).

Leptin is a multifunctional cytokine. It is secreted primarily by adipose tissue but many other tissues can produce it, including macrophages. Leptin is best known as a regulator of satiety and energy homeostasis (Friedman and Halaas, 1998). It acts as a permissive signal when energy levels are high, as represented by adequate fat stores. However, when energy stores are low, leptin secretion decreases and the orexigenic system is activated in the hypothalamus, causing feelings of hunger (Friedman and Halaas, 1998). Human studies that attempted to reduce food intake by exogenous administration of leptin have been disappointing (Heymsfield et al., 1999). A number of theories were raised to explain this lack of appetite suppression. One theory is that leptin receptors are highly expressed in the satiety centers of the hypothalamus, but in order to bind to these receptors, circulating leptin must pass through the blood brain barrier via a saturable process (Lee et al., 1996). It is possible that the high circulating leptin levels observed in obese individuals do not result in a similar increase in brain leptin. Interestingly, Faouzi *et al.* have shown that specific hypothalamic regions establish a direct contact with the general circulation and thereby display differential patterns of

leptin uptake and responsiveness (Faouzi et al., 2007). Another mechanism potentially explaining the lack of therapeutic benefits of leptin is that individuals may acquire leptin resistance (Van Heek et al., 1997) in a manner similar to insulin resistance, including the disruption of downstream leptin receptor signaling by SOCS proteins (O'Connor et al., 2009).

Leptin has a number of important functions in immunity. It has been shown to induce the production of TNF-α, IL-1β, IL-1RA, IL-R2 and IL-6 as well as that of reactive oxygen species, and to increase phagocytosis in some antigen presenting cells (Dreyer et al., 2003; Gabay et al., 2001; Lam and Lu, 2007). Recently, a role of leptin in the regulation of emotions and depression has been suggested. *db/db* mice lacking a functional long form of the leptin receptor showed delayed recovery from LPS- or hypoxia-induced social withdrawal (Johnson et al., 2007; O'Connor et al., 2005). This delayed recovery was accompanied by a failure to upregulate the anti-inflammatory cytokines IL-1RA and IL-1R2. Administration of exogenous leptin was also found to relieve anhedonia, demonstrating its potential to act as an antidepressant (Lu et al., 2006). Given these numerous functions of leptin, it is likely that its implication in T2D will be the subject of many new discoveries.

TNF-α

TNF-α is now recognized as an important modulator of immunity and metabolism, inducing loss of social exploration (Bluthe et al., 2000a), production of acute phase proteins (Gresser et al., 1987) and activation of dendritic cell migration (Cumberbatch and Kimber, 1995). TNF-α is a 27 kDa protein that is processed into a 17 kDa active form. TNF-α has been shown to induce insulin resistance (Hotamisligil et al., 1996; Pickup and Crook, 1998) and to be implicated in the progression of obesity (Spiegelman and Hotamisligil, 1993). Chronic exposure of adipocytes to TNF-α strongly inhibited insulin-stimulated glucose uptake and decreased the phosphorylation of the insulin receptor by insulin (Hotamisligil et al., 1994a; Hotamisligil et al., 1994b). Some controversy exists as to whether TNF-α is a causative agent in T2D. In subjects suffering from impaired glucose tolerance (IGT), TNF-α levels were not elevated by contrast with IL-6 levels (Muller et al., 2002). However, TNF-α receptor knockout mice showed an improved glucose tolerance and increased insulin sensitivity (Hotamisligil et al., 1996). Leptin deficient ob/ob mice with an added p75 TNF-α receptor knockout exhibited improved glucose tolerance (Voros et al., 2004). Additionally, TNF-α is strongly linked with cardiovascular complications which are the leading cause of death in diabetes (Kleinman et al., 1988). TNF-α may accelerate the atherosclerotic process (Lopes-Virella

and Virella, 1996) through an increase in the expression of endothelin-1 (Klemm et al., 1995) and by an alteration of lipid metabolism (Uysal et al., 1997).

IL-1β

Interleukin-1β is a 17.4 kDa protein derived from the cleavage of a 33 kDa inactive precursor by interleukin-1-β-converting enzyme (Cerretti et al., 1992). IL-1β signaling occurs through the evolutionarily conserved MyD88 pathway and the activation of NF-κB. IL-1β is produced by a variety of tissues and cell types including macrophages, neurons, β cells of the pancreas and adipose tissue. IL-1β is known to induce sickness behavior, fever and the secretion of other cytokines (Bluthe et al., 1997). Like TNF-α and leptin, IL-1β has important effects on metabolism. For instance, the activation of IL-1β receptors in hypothalamic neurons caused a marked reduction in food intake (Bluthe et al., 1997; Plata-Salaman, 1994). The functions of IL-1β are counter-regulated in part by competitive inhibition by IL-1RA and IL-1R2 (Bluthe et al., 1992). Importantly, these counter-regulatory mechanisms were deficient in type 2 diabetic *db/db* mice injected with LPS, IL-1β or in hypoxic conditions (Johnson et al., 2007; O'Connor et al., 2005), in contrast with T2D humans who tend to have higher basal serum levels of IL-1RA. Additionally, IL-1β was shown to induce apoptosis in pancreatic β cells. This

finding was first described in T1D but it was also demonstrated that β cell loss in T2D was partially mediated by IL-1β (Bendtzen et al., 1986).

IL-6

IL-6 is a 27 kDa four helix-bundle cytokine with structural similarity to leptin (Somers et al., 1997). The IL-6 receptor is a heterodimer consisting of a gp130 subunit and IL-6R. IL-6 directly affects many tissues including B cells, T cells, megakaryocytes, macrophages, hepatocytes, osteoclasts, blood vessels, heart muscle, neuronal cells and the placenta (Kishimoto et al., 1995). IL-6 is produced mainly by cells of the immune system, skeletal muscle and the liver, but other cells types such as glia and endothelial cells have been reported to produce IL-6 (Kishimoto, 2005). The effects of IL-6 differ according to the target tissues. IL-6 is a key regulator of the acute phase response by the liver following infection. It induces the production of C-reactive protein, haptoglobin, serum amyloid A and fibrinogen (Castell et al., 1988). Like leptin, IL-6 signaling occurs through the MAP kinase and JAK/STAT pathways (Zhong et al., 1994). IL-6 is a potent endogenous pyrogen and augments LPS induced sickness behavior (Bluthe et al., 2000b).

The role of IL-6 in T2D is complex and appears to be tissue-dependent. Circulating levels of IL-6 levels are increased in T2D (Pickup et al., 1997). A chronic

overexpression of IL-6 appears to reduce the action of insulin like growth factor in mice displaying growth defects. This effect was partially neutralized by the administration of anti-IL-6 receptor antibodies (De Benedetti et al., 1997). In addition, IL-6 has been shown to promote insulin resistance in hepatocytes through the activation of STAT-3 (Senn et al., 2002). This mechanism was further elucidated by the finding that insulin resistance in hepatocytes was mediated by SOCS-3 and that mTOR played a critical role in SOCS-3 upregulation (Kim et al., 2008). Additionally, Cai et al. (2005) showed that T2D could be induced in mice by chronic activation of NF-κB in the liver or a high fat diet. These chronic inflammatory conditions induced steatosis of the liver and an increased production of proinflammatory cytokines by hepatocytes, including IL-6. Importantly, insulin resistance could be significantly improved by treatment with IL-6 neutralizing antibodies or salicylate (Cai et al., 2005). These findings suggested a causative role for IL-6 in the development of T2D. However, mice with a targeted deletion of IL-6 developed mature-onset insulin resistance, obesity and leptin resistance (Wallenius et al., 2002b). It was speculated that the reason for this contrary finding was that the action IL-6 is tissue-dependent. Indeed, the local administration of IL-6 into the brains of IL-6 deficient mice partially improved the aforementioned symptoms but it had no effect when administered into the brain of wild type control animals (Wallenius et al.,

2002a). The importance of tissue specificity was further emphasized by the finding that IL-6 enhanced insulin-stimulated glucose disposal and improved glucose metabolism in humans through the activation of AMPK, likely in skeletal muscle (Carey et al., 2006). IL-6 is clearly an important cytokine in the regulation of immunity and metabolism and it may be an important player in the development and complications of T2D. Further research will be necessary to clarify the absolute impact of IL-6 in T2D.

Cytokines Resistance

Counter-regulations are critical to maintain homeostasis. One of the most important mechanisms of hormone/cytokine counter-regulation is mediated by the SOCS family of proteins. While investigating the downstream signaling cascade of IL-6, Kishimoto *et al.* (2005) discovered a protein that they called STAT-induced STAT inhibitor or SSI-1. As the name indicates, this protein inhibited IL-6-mediated STAT activation and was itself induced by activation of STAT (Naka et al., 1997). SSI-1 was later found to be part of a larger family of proteins which are now entitled SOCS proteins. This family of proteins contains an SH-2 domain that can interact with several receptors at phosphotyrosine residues to block signal transduction. Some of the important signaling molecules regulated by the SOCS are insulin, IGF-1, leptin, IL-6, IL-4 and IL-10 (Kim et

al., 2008; Mooney et al., 2001; O'Connor et al., 2007). Chronic activation of the aforementioned receptors can induce a state of functional resistance to the ligand responsible for that specific receptor's activation. In a case of ligand-dependent chronic activation, ligand-specific receptor insensitivity occurs, as does spillover insensitivity to other receptor pathways, due to SOCS upregulation. This finding has led to the speculation that T2D was caused by chronic over expression of SOCS proteins (Mooney et al., 2001). Work by our group indicated that hyperglycemia and hyperinsulinemia contributed to insulin resistance by activation of the nutrient sensing mTOR pathway (Hartman et al., 2004). Kim and colleagues extended this finding by showing that inhibition of mTOR by rapamycin blocked IL-6-induced SOCS protein-mediated insulin resistance (Kim et al., 2008). Recently, it was demonstrated that T2D-dependent upregulation of SOCS proteins negatively impacted the efficacy of the anti-inflammatory cytokine IL-4 to induce IL-1RA by (O'Connor et al., 2007), adding to the growing body of evidence that implicates the SOCS proteins as key immune and metabolic regulators. Additionally, there now appears a direct mechanistic path to explain how dysregulation in certain immune pathways can adversely impact metabolic systems and vice versa.

Anti-inflammatory Interventions

The treatment of diabetes was very limited until the discovery and purification of insulin by Banting and Best in 1921. However, before the discovery of insulin, Ebstein showed that daily consumption of high doses of salicylates greatly reduced glucose elimination in the urine (Ebstein, 2002). There were a handful of additional studies that explored this finding further, as reviewed by Shoelson (Shoelson et al., 2006), but these early promising results were overshadowed by the tremendous success of insulin in the treatment of hyperglycemia. Recently, the emergence of the idea that T2D is an inflammatory disease has led to reexamining the use of anti-inflammatory agents in the treatment of T2D complications. Interestingly, some of the medications currently used as anti-hyperglycemic agents, such as the PPARγ agonist rosiglitazone, may actually mediate at least part of their action through anti-inflammatory effects (Mohanty et al., 2004). Likewise, HMG-CoA reductase inhibitors (statins) have long been known to reduce cardiovascular disease, a serious complication of T2D, by reducing endogenous cholesterol production. Recently, a growing body of evidence suggests that statins exert potent anti-inflammatory effects (Ridker et al., 2001). Metformin, one of the most commonly prescribed drugs in the treatment of T2D has been shown to act as an anti-inflammatory agent by activating AMPK (Hattori et al., 2006). Use of metformin is

interesting because AMPK is a key local and systemic metabolic regulator. In addition, these findings underscore the degree of integration between metabolism and immunity. Finally and very recently, IL-1RA has shown promise in improving glycemia, β-cell secretory function and reducing markers of systemic inflammation (Larsen et al., 2007). By expanding our understanding of T2D, we have increased the therapeutic options available to the individual with diabetes, and have, in some ways, returned to Ebstein's original observations with the archetypal anti-inflammatory aspirin (Shoelson et al., 2006).

Conclusions

Inflammation can be viewed as a homeostatic model with pro- and anti-inflammatory aspects. Proinflammatory cytokines are necessary in order to mount an initial effective immune response. However, this proinflammatory reaction must be balanced by an appropriate anti-inflammatory rejoinder in order to effectively direct the adaptive immune response and to avoid excessive damage to healthy tissues. Furthermore, the immune response must be in balance with the metabolic supplies of the organism. Immune and metabolic pathways are deeply intertwined and require synchronicity in order to promote organismal survival ((Guest et al., 2006). T2D is a key

example of what happens when balance goes awry. Neither cytokine nor hormonal networks function in isolation so it is likely that many important future contributions to our understanding of T2D will be found by examining the complicated interaction and temporal variations of immune/metabolic balance. While tremendous strides have been made in understanding the nature of T2D and its complications, much more work needs to be done to improve the lives of these individuals living with the all too familiar quartet of swelling, redness, pain and heat.

2.4 Intensive Insulin Therapy

Intensive insulin therapy (IIT) differs from conventional insulin replacement in that it requires close monitoring of blood glucose and more frequent administration of insulin. This type of treatment, which includes the use of insulin pumps or basal-bolus injections, mimics natural insulin secretion patterns and is more effective in maintaining euglycemia than is conventional treatment. However, use of IIT significantly increases the risk of hypoglycemia. Hypoglycemia can lead to recurrent physical and psychosocial morbidity, and sometimes death (Cryer, 2004). IIT has been the standard care for T1D for more than a decade. This has expanded to include the treatment of T2D, as its incidence and severity have increased.

Hospitalized patients in medical or surgical intensive care units (ICU) often have high blood glucose, even if they do not have diabetes. In the past, this was generally viewed by physicians as a response to other conditions and not treated. However, a 2001 study revealed that maintaining strict glycemic control through IIT reduced mortality by nearly 50% and significantly reduced associated morbidities such as renal failure and need for mechanical ventilation in ICU patients (van den Berghe et al., 2001). Glycemic control became widely accepted in critical care situations and likewise, the incidence of hypoglycemia in critically ill patients increased. There was a six-fold increase in the incidence of severe hypoglycemia and a five-fold increase in multiple episodes of hypoglycemia in patients treated with IIT (Cryer, 2006). The underlying illness in ICU patients, combined with their inability to consciously respond to classical neuroglycopenic symptoms, further increases the risk of detrimental effects or fatality from hypoglycemia (Cryer, 2006; Cryer, 2006).

There are long-term effects of hypoglycemia as well. Young children who have had major hypoglycemic events such as seizures scored lower on tests of IQ and presented a tendency to make more errors on complicated tasks later in life. In adults, hypoglycemia causes a lower performance IQ (Northam et al., 2001).

2.5 Hypoglycemia

Hypoglycemia is the most common result of drugs used to treat diabetes. In humans, the normal range of fasting plasma glucose concentration is approximately 70-110 mg/dl (3.9-6.1 mmol/L) and it is maintained within a narrow range. Glucose levels < 55 mg/dl (3.0 mmol/L) with symptoms that are relieved promptly after the glucose level is raised document hypoglycemia (Braunwald, 2001).

The maintenance of normal plasma glucose levels is critical to survival and involves a network of hormones, neural signals and glucose utilization by tissues other than the brain. Central and peripheral sensors detect hypoglycemia and coordinate neuroendocrine and autonomic responses to mobilize glucose (Bolli and Fanelli, 1999). The primary defense against hypoglycemia is a decrease in insulin secretion and an increase in glucagon secretion. Secondarily, increased sympathoadrenal outflow stimulates secretion of catecholamine hormones (epinephrine, norepinephrine) from the adrenal medulla (Cryer, 2006). Catecholamines act to suppress insulin secretion, stimulate hepatic/renal gluconeogenesis, and inhibit peripheral glucose use (Rizza et al., 1979). The role of the catecholamine response becomes even more important when the glucagon response is absent or diminished, as seen in certain clinical syndromes and during prolonged hypoglycemia (De Feo et al., 1991).

Increased sympathoadrenal outflow from the brain generates neurogenic (autonomic) symptoms including tremor, shaking and anxiety; and cholinergic symptoms such as sweating, hunger and paresthesia (Braunwald, 2001). These symptoms are similar to those observed in the classic sickness response to a pathogenic infection (Dantzer, 2009).

2.6 Hypoglycemia in Diabetes

Hypoglycemia is most commonly caused by the treatment of diabetes. People with T1D suffer an average of two episodes of symptomatic hypoglycemia per week. An estimated 2~4 % of people with T1D die as a result of hypoglycemia each year (Musselman et al., 2003; Leckie et al., 2005; Frier, 2004). Hypoglycemia affects persons with T2D as well as those with T1D because both require insulin treatment and develop insulin deficiency, which is why hypoglycemia is called a fact of life for people with T1D and T2D (Braunwald, 2001).

Hypoglycemia can lead to recurrent physical and psychosocial morbidity, and sometimes death (Cryer, 2008; Frier, 2004; Leckie et al., 2005). Strict glycemic control is essential for the treatment of diabetes and in reducing morbidity and mortality in critically ill patients with T1D and many with T2D. It is known that use of IIT

significantly increases the risk of hypoglycemia (van den Berghe et al., 2001). Hypoglycemia is not only the major barrier but also the limiting factor to achieving euglycemia and its associated benefits in these populations through the use of IIT (Cryer, 2008; Service, 1995).

2.7 Hypoglycemia and Depression

In a study of 1200 hypoglycemic patients, Gyland found that 77% of the patients showed several symptoms of depression, and 62% showed worrying and anxiety (Gyland, 1953). More recently, positron emission tomography (PET) scans have verified that glucose metabolism is often reduced in the brains of patients suffering from depression. Gold and colleagues has examined and reviewed the non-cognitive impact of insulin-induced hypoglycemia (Gold et al., 1993; Gold et al., 1995; Gold et al., 1997). In non-diabetic participants, they found that hypoglycemia caused mood changes including a reduction in hedonic tone and energetic arousal and an increase in tense arousal. They also noted that tense-tiredness persisted for at least 30 min after restoration of euglycemia (Gold et al., 1993). Tense-tiredness may be of particular relevance to T1D in that it is a mood where fatigue is mixed with nervousness, tension or anxiety and often underlies depression (Diedrich et al., 2002; Gold et al., 1995). T1D is linked to an

increase in mental health and mood difficulties including anxiety, depression and social withdrawal (Delamater AM, ; Grey et al., 1995; Lloyd et al., 2000; Lloyd et al., 2003; Peyrot and Rubin, 1997; Shaban et al., 2006; Steinsdottir et al., 2008). Withdrawn children are anxious, lonely, fail to exhibit age-appropriate interpersonal problem-solving skills and are deficient in social skills and social relationships (Silverstein et al., 2005). It is important to note that social withdrawal is a component of these behaviors including tense arousal, fatigue and anxiety (Goodman et al., 2006a).

2.8 Adrenergic Regulation of Hypoglycemia and Depression

It has been reported that communication exists between adrenergic regulation and energy metabolism. Schwab et al. (1993; 2004) showed a significant negative correlation between a hypoglycemia incidence (blood glucose < 50 mg/dl) and β-2-AR densities on lymphocytes and β-2-AR densities were decreased in patients with symptomatic hypoglycemia unawareness. Hagstrom-Toft et al. (1998) showed the importance of β-adrenergic regulation of lipolysis in human skeletal muscle by reporting that nonselective and β-2-selective blocking agents inhibited the hypoglycemia-induced lipolysis.

The neurophysiology of depression has been studied extensively in man and in animal models (Dantzer et al., 2008; Dantzer, 2009; Krishnan and Nestler, 2008). Acute stress is associated with a variety of physiological responses including the activation of the hypothalamic–pituitary–adrenal axis (HPA – axis) with a concomitant peripheral release of adrenocorticotropic hormone (ACTH), Epi and cortisol, a significant increase in centrally-controlled peripheral sympathetic nervous system tone, and the activation of a variety of neurochemical systems in the CNS. One of the most critical of these systems is the noradrenergic nucleus in the locus coeruleus (LC) (Usdin et al., 1976). This region controls noradrenergic tone and activity throughout the midbrain and in important forebrain areas including the cortex (Usdin et al., 1976). The LC has been shown to be critical in many regulatory functions including the regulation of affect, irritability, locomotion, arousal, attention and startle. Chronic stress such as footshock or handling results in altered β and α2-adrenergic receptor functioning (decreased α2 receptors and the less efficient coupling of β and α2 receptors to adenylate cyclase) in many brain regions (Kaan et al., 1996). These changes are thought to reflect homeostatic changes resulting from the increased activity of the adrenergic and noradrenergic systems mediating the CNS stress response. Adrenergic and noradrenergic systems and their receptors are involved in the mediation and recovery from the observed behavioral

changes following inescapable shock (Ferguson, 1978). It is of interest to note that under certain conditions tricyclic antidepressant medications attenuate the effects of inescapable shock in animals (Kitada et al., 1981; Petty and Sherman, 1980; Sherman et al., 1979; Sherman and Petty, 1980).

CHAPTER 3: BLOCKING OF β-2 ADRENERGIC RECEPTORS HASTENS RECOVERY FROM HYPOGLYCEMIA-ASSOCIATED SOCIAL WITHDRAWAL[2]

3.1 Abstract

Objective: Hypoglycemia is associated with a variety of adverse behaviors including fatigue, confusion and social withdrawal. While these clinical symptoms are well characterized, the mechanism of their cause is not understood. Herein, we investigated how insulin-induced hypoglycemia causes social withdrawal.

Research design and methods: Male 8-12-week-old C57BL/6J mice were injected intraperitoneally (IP) with or without and/or insulin, norepinephrine (NE) and epinephrine (Epi), terbutaline and butoxamine with subsequent measurement of blood glucose, social withdrawal and plasma catecholamines.

Results: Insulin generated significant hypoglycemia with blood glucose nadirs at 0.75h post-injection of 64 ± 4 and 48 ± 5 mg/dl for 0.8 and 1.2 units/kg of insulin, respectively. Insulin (0.8 or 1.2 units/kg) caused near total social withdrawal at 0.75h

[2] Park, M. J., Guest, C. B., Barnes, M. B., Martin, J., Ahmad, U., York, J. M., et al. (2008). Blocking of β-2 adrenergic receptors hastens recovery from hypoglycemia-associated social withdrawal. Psychoneuroendocrinology, 33(10), 1411-1418

with full recovery not occurring until 4h (0.8 units/kg) or 8h (1.2 units/kg) post-insulin injection. Insulin also caused a marked elevation in plasma catecholamines. Basal 12h fasting NE and Epi were 287 ± 38 and 350 ± 47 pg/ml, respectively. Insulin at 0.8 units/kg increased plasma NE and Epi to 994 ± 73 and 1842 ± 473 pg/ml, respectively. Administration of exogenous NE or Epi caused social withdrawal similar in magnitude to insulin. Importantly, administration of the β-2 adrenergic receptor agonist terbutaline also caused social withdrawal while administration of the β-2 adrenergic receptor antagonist butoxamine blocked NE-induced social withdrawal. Finally, butoxamine blocked insulin-induced social withdrawal.

Conclusions: These data demonstrate that hypoglycemia-associated social withdrawal is dependent on catecholamines via a β-2 receptor-mediated pathway.

3.2 Introduction

Hypoglycemia (defined as blood glucose less than 60 mg/dl) is the most common complication of type 1 diabetes (T1D) in childhood (Daneman, 2006; Shalitin and Phillip, 2007). It occurs when the administered dose of insulin exceeds the insulin requirement and is especially common in tightly controlled patients (Shalitin and Phillip,

2007). Symptoms of hypoglycemia may be adrenergic in origin due to Epi release or related to neuroglycopenia (Hoffman et al., 1997; Hoffman, 2007; Korytkowski et al., 1998; Service, 1995; Ste Marie and Palmiter, 2003). The adrenergic symptoms include: tremor, pallor, rapid heart rate, palpitations and diaphoresis (Binder and Bendtson, 1992; Bolli, 1997; Korytkowski et al., 1998). Neuroglycopenic symptoms range from fatigue, lethargy, headache, drowsiness and behavior change to seizures, unconsciousness and coma (Binder and Bendtson, 1992; Hoffman, 2007). Symptoms of hypoglycemia are classified as mild, moderate or severe (Hoffman, 2007). Mild hypoglycemia is associated with adrenergic symptoms and mild neuroglycopenic symptoms such as headache and behavior change (Frier, 2004; Hoffman, 2007). In addition, mild symptoms are generally recognized by the patient and can be adequately treated without the intervention of a second person (Frier, 2004). Moderate and severe cases require a second person's assistance (Davis et al., 1998; Frier, 2004).

The brain is highly glucose-dependent, but it can neither synthesize glucose nor store significant amounts of it (Rao et al., 2006). With the more frequent use of intensive therapies for T1D, symptomatic hypoglycemia has increased in incidence with more than 17% of individuals noting a hypoglycemic episode during a year's treatment time (Feingold, 1991). Severe hypoglycemia, particularly that presenting with seizure or coma,

may result in permanent impairment especially in children less than 5 years of age (Cryer, 2008). In addition, repeated episodes of hypoglycemia can negatively impact brain development and learning (Cryer, 2008). Even isolated acute episodes of mild hypoglycemia can transiently impair attention, mentation and memory (Northam et al., 2001).

Type 1 diabetes is, also, linked to an increase in mental health and mood difficulties including anxiety (McAulay et al., 2006), depression (Hislop et al., 2008) and social withdrawal (Delamater AM,). Withdrawn children are anxious, lonely, fail to exhibit age-appropriate interpersonal problem-solving skills and are deficient in social skills and social relationships (Silverstein et al., 2005). In 2000, the International Society of Pediatric and Adolescent Diabetes (ISPAD) Consensus Guidelines stated that ‘‘psychosocial factors are the most important influences affecting the care and management of diabetes’’ and these recommendations were reiterated in the 2006/2007 ISPAD guidelines. Unfortunately, very little is known about how T1D causes neurocognitive, psychosocial and behavioral difficulties and how they are regulated in the body either chronically or acutely. Therefore, we sought to investigate the acute mechanism by which insulin-induced hypoglycemia causes the adverse behavior of social withdrawal using a mouse model.

3.3 Materials and methods

Materials

All reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except for Humalin R (insulin), which was purchased from Eli Lilly (Indianapolis, IN).

Animals

All animal care and use was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council). C57BL/6J mice were bred in-house from mice purchased from The Jackson Laboratory. Mice were group housed (4—8) in standard shoebox cages (17.15 cm X 28 cm) in a temperature (23.8ºC) and humidity (45—55%) controlled environment, with a 12-h/12-h dark/light cycle (0800h to 2000 h). Mice were fed pelleted food (NIH 5K52; LabDiet; Purina Mills) and water *ad libitum*. Male 8—12-week-old animals were used for all experiments. Animals were administered epinephrine (Epi), norepinephrine (NE) and insulin at the indicated concentrations via IP injection. Butoxamine and terbutaline were administered IP at 5 mg/kg.

Blood glucose

Blood was collected from the tail as described previously (Hartman et al., 2004). Briefly, blood glucose levels were measured using a One Touch Ultra glucometer (Johnson & Johnson, Milpitas, CA) per the manufacturer's instructions. In brief, mice were placed in a very shallow shoebox sized container (17.15 cm X 28 cm X 4 cm) such that the tail was exposed. The tip of the tail was then secured against the top of the container, snipped and blood drawn. Blood glucose was measured on the same mice utilized in the social withdrawal experiments.

Social withdrawal

Social withdrawal was measured as previously described (Hartman et al., 2004). In brief, juvenile and adult mice were individually housed for 18h prior to experimentation. A novel 3-4-week-old conspecific juvenile mouse (challenge mouse) was then confined to a 7.62 cm X 7.62 cm wire mesh enclosure (with a perforated steel top and bottom) which was placed in the corner of the home cage of the adult mouse (test mouse) for 5 min immediately prior to and at the indicated times after treatment (n = 3-4). A novel juvenile was supplied for each interaction at every time point. Interaction (nose contact) between test and challenge mouse was video-recorded. Time spent by the test

mouse in exploratory behavior was determined from video records. To control for mouse-to mouse variability in baseline activity and to allow comparison of relative changes in exploration levels, a pre-exposure (0 h) measurement was used as an internal control for each mouse. Results are expressed as percentage of baseline measurement and shown as means ± SEM For all behavior experiments, mice were fasted for 12h then pre-injected IP (where indicated) with the described agonist, antagonist or saline 0.5h prior to IP insulin or IP saline administration. Unrestricted access to food was provided 0.75h after agonist or insulin administration. Social exploration was measured at the time points indicated with the clock starting after insulin delivery. In experiments without insulin, the starting point was after agonist or saline delivery. All experiments were performed under red light, during the dark cycle 1h into darkness.

Movement

Movement was measured in a four arm, black, Plexiglas crossmaze (arms = 27.5 cm in length X 8 cm in width X 10 cm wall height: central platform = 8 cm X 8 cm) by methods previously described (Ragozzino et al., 1998). In brief, mice were placed on the center platform at the times indicated. Movement, as assessed by arm entries, was recorded over a 5 min period (from video records). The mouse was required to have all

four legs in the arm for an arm entry to have occurred.

Plasma catecholamine analysis

After the indicated treatments, mice were anesthetized with sodium ketamine hydrochloride:xylazine hydrochloride (80 mg/ml:12 mg/ml, ketamine:xylazine) at 1.5 ml/kg body weight and blood removed from the left ventricle. Blood was collected into chilled heparinized centrifuge tubes and spun at 9300 X g for 8 min. Plasma was aspirated and stored at -80ºC. Catecholamines were determined from plasma by reverse-phase high-performance liquid chromatography (HPLC). Solid phase extraction was with aluminum oxide (Bioanalytical Systems, West Lafayette, IN) and elution was in 0.2 N perchloric acid. Dihydroxybenzylamine was used as an internal standard to determine extraction efficiency. Electrochemical detection (ESA, Chelmsford, MA) utilized a 150 X 2 mm C18 (3 mm) Hypersil column (Keystone Scientific, Bellfonte, PA) fitted with a 2 mm C18 (3 mm) Hypersil javelin guard column (Keystone Scientific). Mobile phase (pH 3.0) was 75 mM NaH2PO4, 1.7 mM 1-ocatanesulfonic acid, 25 mM Na2EDTA, 7% (vol/vol) acetonitrile and 0.1% (vol/vol) triethylamine. The interassay coefficient of variation was less than 3%.

Statistical analysis

Data are presented as mean ± SEM and were analyzed by two- or three-way ANOVA depending on the experimental design with repeated measurements in the time factor as applicable. Post hoc comparisons of individual group means were carried out with the Tukey test (SAS Institute, Cary, NC). Statistical significance was denoted at $p < 0.05$.

3.4 Results

Insulin-induced hypoglycemia is associated with social withdrawal

Table 3.1 demonstrates that when C57BL/6J mice were withheld food for 12h, blood glucose ranged from 117 ± 6 to 131 ± 11 mg/dl. When mice were injected IP with insulin, blood glucose fell. Blood glucose 0.75h after 0.4, 0.8 or 1.2 units/kg of insulin was 99 ± 14 mg/dl ($p = 0.029$), 64 ± 4 mg/dl ($p = 0.001$) or 48 ± 5 mg/dl ($p = 0.0008$), respectively compared to control (151 ± 13 mg/dl). Food was made accessible to the mice 0.75h post injection. At 8h post insulin injection, and 7.25h post return to unrestricted food access, blood glucose ranged from 189 ± 17 to 200 ± 6 mg/dl in insulin treated and control animals. **Fig. 3.1** shows the impact of insulin administration on social

exploration. At 0.75h after IP insulin injection, social withdrawal was nearly complete in mice treated with 0.8 and 1.2 units/kg insulin, demonstrating a 91 ± 11% ($p = 0.0001$) and 96 ± 5% ($p = 0.0001$) loss in social exploration, respectively. Insulin delivered at 0.4 units/kg did not impact social exploration. In addition, recovery from insulin-induced social withdrawal took 4 and 8h to recovery from after 0.8 and 1.2 units/kg insulin, respectively. Finally, arm entries into a plus maze were examined to assess mouse mobility after administration of 0.8 units/kg insulin. As with social withdrawal, 0.75h after insulin injection, arm entries in insulin-treated mice were reduced {44 ± 10 vs 14 ± 4 ($p = 0.038$)}. After 3h (for arm entries), insulin-treated mice had fully recovered. Taken together, these findings indicate that insulin-induced hypoglycemia is associated with social withdrawal and loss of movement.

Catecholamines cause social withdrawal

Fig. 3.2A demonstrates that 0.8 units/kg insulin IP induced a marked elevation in plasma NE and Epi. At 0.75h after insulin, NE was increased compared to control: 994 ± 73 pg/ml vs 439 ± 50 pg/ml ($p = 0.001$). At 120 min after insulin, NE returned to near control levels: 994 ± 73 pg/ml vs 351 ± 54 pg/ml ($p = 0.052$). After insulin (0.75 h), Epi increased to 2184 ± 833 pg/ml vs 390 ± 11 pg/ml ($p = 0.089$) and was significantly

elevated at 120 min post insulin, 1842 ± 472 pg/ml vs 351 ± 144 pg/ml ($p = 0.01$). To determine the impact of catecholamines on social withdrawal, social exploration was examined. **Fig. 3.2B** shows that when NE was administered IP at 1.0, 1.5 or 2.0 mg/kg, social exploration was significantly curtailed 0.5h after injection {63 ± 5% ($p = 0.009$), 38 ± 9% ($p < 0.001$) or 19 ± 3% ($p < 0.001$), respectively}. Recovery from NE-induced social withdrawal occurred at 2h for NE at 1.0 mg/kg and at 4h for NE at 1.5 and 2 mg/kg. **Fig. 3.2C** demonstrates that Epi was a more potent inducer of social withdrawal. At 0.25, 1.0 and 1.5 mg/kg, Epi caused social exploration to fall to 44 ± 4% ($p = 0.0002$), 27 ± 3% ($p < 0.0001$) and 24 ± 2% ($p < 0.0001$) of control, respectively, 0.5h after administration. Recovery occurred in 2, 4 and 12h after 0.25, 1.0 and 1.5 mg/kg Epi, respectively. Taken together, these findings indicate that catecholamines cause social withdrawal.

β-2 adrenergic receptor stimulation causes social withdrawal which β-2 adrenergic receptor antagonism prevents

To determine if catecholamine-dependent social withdrawal was mediated by the β-2 adrenergic receptor, β-2 agonism was performed using the β-2 agonist terbutaline (Ito et al., 2006; Podojil et al., 2004; Thaker et al., 2006). **Fig. 3.3A** shows that when

terbutaline was administered IP at 5.0 mg/kg, social withdrawal occurred similar to that seen with 1.5 mg/kg NE (30 ± 3% vs. 35 ± 7% at 0.5 h), (43 ± 11% vs. 51 ± 14% at 2 h), (67 ± 7% vs. 80 ± 1% at 4 h). Importantly, when the β-2 antagonist butoxamine (Junker et al., 2002; Kaan et al., 1996) was administered IP at 5.0 mg/kg to mice just prior to NE injection (1.5 mg/kg), NE-dependent social withdrawal was completely blocked. Taken together, these findings indicate that catecholamine-dependent social withdrawal is mediated by the β-2 adrenergic receptor.

Butoxamine blocks insulin-induced social withdrawal

Table 3.2 demonstrates that when C57BL/6J mice were withheld food for 12 h, blood glucose ranged from 137 ± 15 to 149 ± 17 mg/dl. When mice were injected IP with insulin (0.8 units/kg) or insulin (0.8 units/kg) + butoxamine (5 mg/kg), blood glucose fell to 67 ± 4 mg/dl ($p < 0.0001$) or 78 ± 6 mg/dl ($p = 0.0004$), respectively, compared to control (150 ± 10 mg/dl). At 8h post insulin injection and 7.25h post return to unrestricted food access, blood glucose ranged from 202 ± 13 to 231 ± 7 mg/dl in insulin treated and control animals. Butoxamine did not alter the hypoglycemic response to insulin. **Fig. 3.4A** shows the impact of butoxamine administration on social exploration. At 0.75h after IP insulin injection, social withdrawal was nearly complete demonstrating

a 96 ± 6% ($p = 0.0007$) loss in social exploration. Importantly, butoxamine completely blocked the effect of insulin-induced hypoglycemia on social withdrawal while the pan-α blocker phentoloamine and β-1 specific antagonist metoprolol did not (**Fig. 3.4B**). Taken together, these findings indicate that insulin-induced hypoglycemia-dependent social withdrawal is mediated by the β-2 adrenergic receptor.

3.5 Discussion

We have previously shown that in mouse models of T1D and T2D social withdrawal induced by innate immune activation is exaggerated and prolonged (Lin et al., 2007). In diabetic mice administered the toll-like receptor 4 (TLR-4) agonist lipopolysaccharide (LPS), prolonged immune-activated social withdrawal appeared dependent on hyperglycemia (Lin et al., 2007). This is likely due to the impact of hyperglycemia on macrophages because hyperglycemia augments LPS-induced pro-inflammatory cytokine production by macrophages via a pathway requiring p38 map kinase (Sherry et al., 2007). In general, social withdrawal as part of classical sickness symptoms is caused by innate immune activation (Dantzer, 2004) and is dependent on pro-inflammatory cytokines, especially TNF α and IL-1 β, and their impact in the brain

(Dantzer et al., 2008).

As shown in the results for Fig. 3.1, insulin-induced hypoglycemia causes social withdrawal and reduced mouse movement. When insulin is administered at 1.2 units/kg, blood glucose nadirs at 48 mg/dl (Table 3.1) 0.75h after insulin injection, which corresponds with nearly complete social withdrawal. Interestingly, hypoglycemia-associated social withdrawal took 8h to fully recover, indicating a significant behavioral impact of hypoglycemia extending well beyond the acute event. This phenomenon should not be surprising because hypoglycemia triggers a variety of bioactive compounds that raise blood glucose. These include catecholamines, glucagon, growth hormone and cortisol. Metabolically, these agents stimulate glucose production initially through glycogenolysis and then later through gluconeogenesis, decreased muscle glucose storage/oxidation and use of alternative fuels (Hoffman, 2007). Catecholamines, especially, are key to the early glucose rise in T1D because disease-based loss of pancreatic islet cells also disrupts the ability of the pancreas to produce glucagon (Brown et al., 2008).

Insulin-induced hypoglycemia was also associated with decreased mouse movement as measured by arm entries in a cross-maze. When examined as a percentage (at 0.8 units/kg insulin), loss of social exploration (at 0.75 h) was greater (91%) than loss

of movement (68%). This indicates that loss of social exploration may not just be due to a simple loss of activity. In general, severe insulin-induced hypoglycemia lowers brain ATP stores that can take up to 3h to fully recover, if the hypoglycemia is serious enough to cause coma as documented by EEG (Agardh and Rosen, 1983). When insulin was used to drop blood glucose in diabetic humans with from ~180 mg/dl to ~40 mg/dl within 1 h, adrenergic symptoms as measured by pulse returned to normal 1h after the pulse peaked at 1h post-insulin administration (Deacon et al., 1977). Unfortunately, in both animals and humans, little has been reported regarding recuperation from insulin-induced hypoglycemia and almost nothing is known about recovery from adverse behaviors associated with insulin-induced hypoglycemia. Most work has focused on how to effectively and rapidly restore blood glucose and other metabolic indicators of hypoglycemia and correlating return of these biomarkers to normal as resolution (Pratley and Salsali, 2007). Only in severe coma-inducing hypoglycemia does the brain tend to be examined, but in these studies behavior and behavioral recovery is ignored.

Gold et al. (1995, 1997) have examined and reviewed the non-cognitive impact of insulin-induced hypoglycemia (Gold et al., 1995; Gold et al., 1997). In non-diabetic participants, they found that hypoglycemia caused mood changes including a reduction in hedonic tone and energetic arousal and an increase in tense arousal. They also noted

that tense-tiredness persisted for at least 30 min after restoration of euglycemia (Gold et al., 1993). Tense-tiredness may be of particular relevance to T1D in that it is a mood where fatigue is mixed with nervousness, tension or anxiety and often underlies depression (Goodman et al., 2006; Lustman and Clouse, 2007). It is important to note that social withdrawal is a component of these behaviors including tense arousal, fatigue and anxiety (Goodman et al., 2006). In addition, there may be a stratification of hypoglycemia-associated behaviors because we found that peak social withdrawal was more severe than peak loss of movement.

Another question Fig. 3.1 poses is whether insulin itself, not insulin-induced hypoglycemia, causes the social withdrawal observed. Gold et al. (1995, 1997) found that the mood disturbances they observed occurred in the insulin-induced hypoglycemia subjects and not those exposed to hyperinsulinemic glucose clamp. In addition, we have shown that in a mouse model of T1D, insulin does not induce social withdrawal, but appears to improve social exploration in hyperglycemic mice, especially if insulin is administered ICV (Lin et al., 2007).We have also shown that in non-diabetic and T2D mice that IGF-I does not impact baseline social exploration (Johnson et al., 2005).

Fig. 3.2A demonstrates that the insulin dose administered was significant enough to up-regulate plasma NE and Epi. These findings indicated that NE or Epi might be

responsible for the social withdrawal seen with insulin-induced hypoglycemia. As Fig. 3.2B and C show, NE and Epi both cause social withdrawal. Interestingly, Epi appears to be a more potent inducer of social withdrawal, being able to cause social withdrawal at one quarter the dose of NE. In addition, the impact of Epi on social withdrawal was significantly longer lasting when both were administered at 1.5 mg/kg. Fig. 3.3 shows that the β-2 adrenergic receptor agonist terbutaline induces social withdrawal and that the β-2 receptor agonist butoxamine completely blocks NE-induced social exploration. Importantly, butoxamine did not raise blood glucose in response to insulin (Table 3.2), suggesting that insulin-induced social withdrawal is not mediated directly by hypoglycemia but by the impact that hypoglycemia has on catecholamines. Together these findings point to β-2 adrenergic stimulation as key to catecholamine-dependent social withdrawal. Critically, the β-2 antagonist butoxamine blocked insulin-induced social withdrawal (Fig. 3.4A), while the pan-α blocker, phentolamine, and the β-1 blocker, metoprolol, did not (Fig. 3.4B). These findings strongly support our contention than hypoglycemia-associated social withdrawal induced by insulin is dependent on catecholamines via a β-2 receptor-mediated pathway. While these finding do not exclude glucagon and/or cortisol/corticosterone as modulators of behavior in insulin-induced hypoglycemia, with regard to social withdrawal, catecholamines appear paramount.

A key question is how NE/Epi causes social withdrawal. Both NE and Epi (as well as terbutaline) are rather polar compounds that do not readily enter the CNS (Goodman et al., 2006). In general, Epi may cause restlessness and apprehension but these feelings in humans are usually ascribed to the effect of Epi on the cardiovascular system, skeletal muscle and/or intermediary metabolism (Goodman et al., 2006). NE is less commonly linked to restlessness and apprehension than Epi (Goodman et al., 2006) and, like Epi, NE is rapidly inactivated by the same enzymes that methylate and oxidatively deaminate Epi (Goodman et al., 2006). In our study, the apparent reason Epi is a more potent inducer of social withdrawal than NE is that Epi is a more effective β-2 adrenergic agonist than NE (Goodman et al., 2006). Support for this contention is that the β-2 selective adrenergic agonist terbutaline caused social withdrawal and, in general, β-2 agonists are more likely to induce feelings of restlessness, apprehension and anxiety (Goodman et al., 2006). Importantly, these behaviors are linked in certain instances to social withdrawal (Goodman et al., 2006). The probable mechanism by which β-2 adrenergic stimulation causes social withdrawal either due to adrenergic agents or insulin-induced hypoglycemia and subsequent catecholamine up-regulation is through ‘‘stress’’-induced hypothalamic NE turnover (Anisman and Sklar, 1979; Weiss et al., 1975) because NE turnover induces social withdrawal in immune-based sickness models

(Marvel et al., 2004). Finally, what causes glucoprivic triggering of noradrenergic neurons in the ventromedial hypothalamus is not clear (Levin, 2007), but nearly one-third of young adults with T1D experience psychological distress and this distress appears linked to hypoglycemia especially in those attempting tighter glucose control with subcutaneous insulin infusion (Hislop et al., 2008). Therefore, the importance of understanding the adverse impact of hypoglycemia on behavior is significant.

3.6 Figures and Tables

Table 3.1: Blood Glucose (mg/dl) after Insulin Injection

Treatment	0 h	0.75 h	8 h
Saline	130 ± 11	151 ± 13	196 ± 4
Insulin 0.4 units/kg	131 ± 11	99 ± 14 *	190 ± 19
Insulin 0.8 units/kg	117 ± 6	64 ± 4 *	200 ± 6
Insulin 1.2 units/kg	124 ± 18	48 ± 5 *	189 ± 17

*$p<0.05$, 0.75h vs. 0h.

Table 3.2: Plasma Glucose (mg/dl) after Insulin (Ins) and Butoxamine (BTX) Injection

Treatment	0 h	0.75 h	8 h
Saline	149 ± 17	150 ± 10	226 ± 17
Butoxamine	139 ± 7	151 ± 14	202 ± 13
Insulin	137 ± 15	67 ± 4 *	224 ± 11
Ins + BTX	142 ± 7	78 ± 6 *	231 ± 7

*$p<0.05$, 0.75h vs. 0h.

Figure 3.1

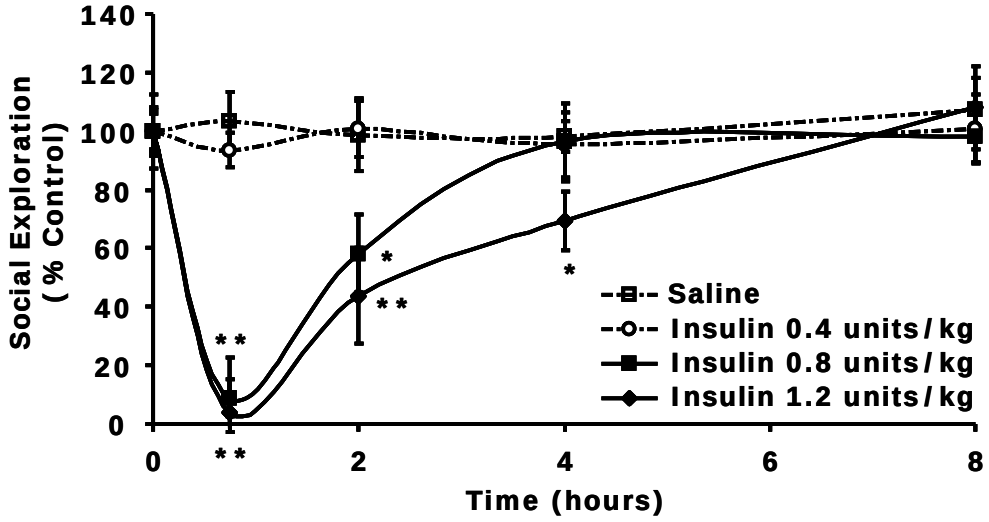
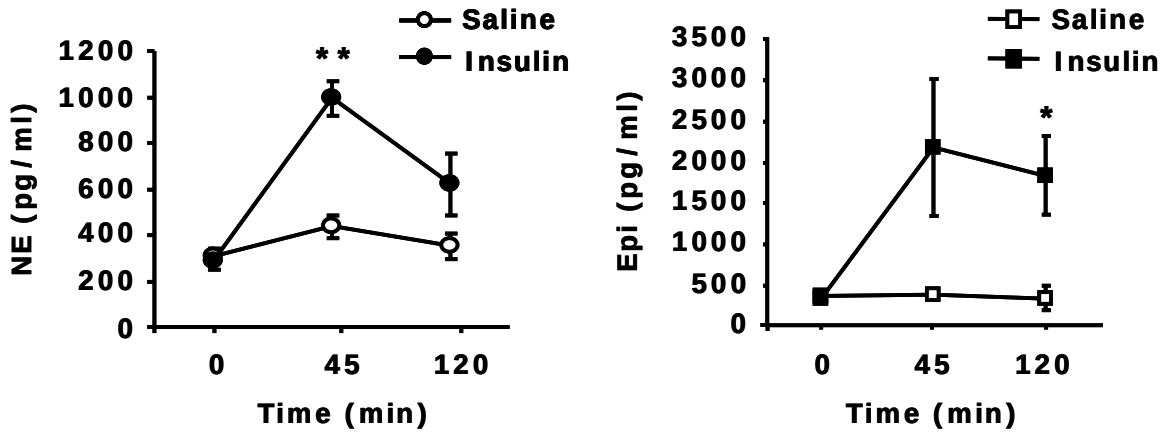


Fig 3.1. Insulin induces social withdrawal. After a 12h fast, C57BL/6J mice were administered either insulin (Insulin) or saline control (Saline) IP as indicated. Social exploration was measured at 0, 0.75, 2, 4 and 8h after insulin delivery. Unrestricted access to food was provided after the 0.75 time point. Results are expressed as percentages of the baseline measurement, means ± SEM; n=3, *P <0.05, **P <0.001 Insulin vs. Saline.

Figure 3.2

(A)


Saline
Insulin
NE (pg/ml)
1200
1000
800
600
400
200
0
**
0
45
120
Time (min)
Saline
Insulin
Epi (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
*
0
45
120
Time (min)

(B)

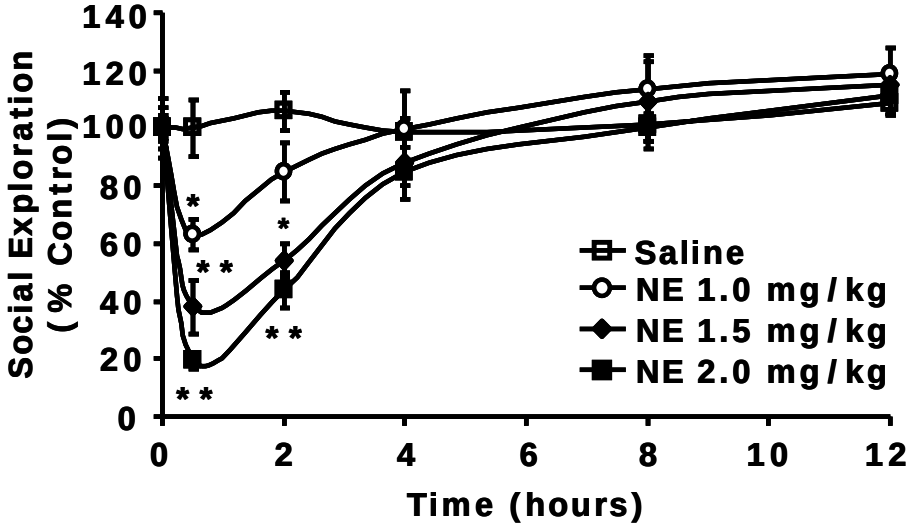

Social Exploration (% Control)
140
120
100
80
60
40
20
0
*
**
*
**
**
Saline
NE 1.0 mg/kg
NE 1.5 mg/kg
NE 2.0 mg/kg
0
2
4
6
8
10
12
Time (hours)

Fig. 3.2, continued

(C)

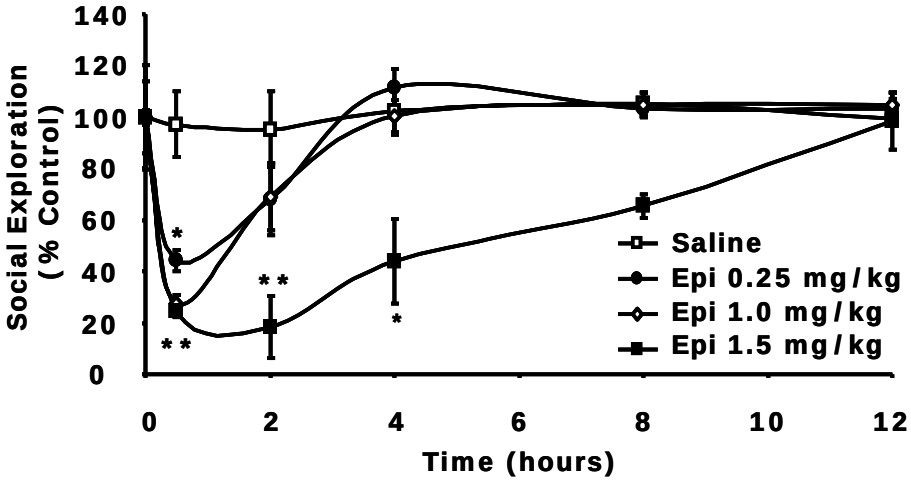
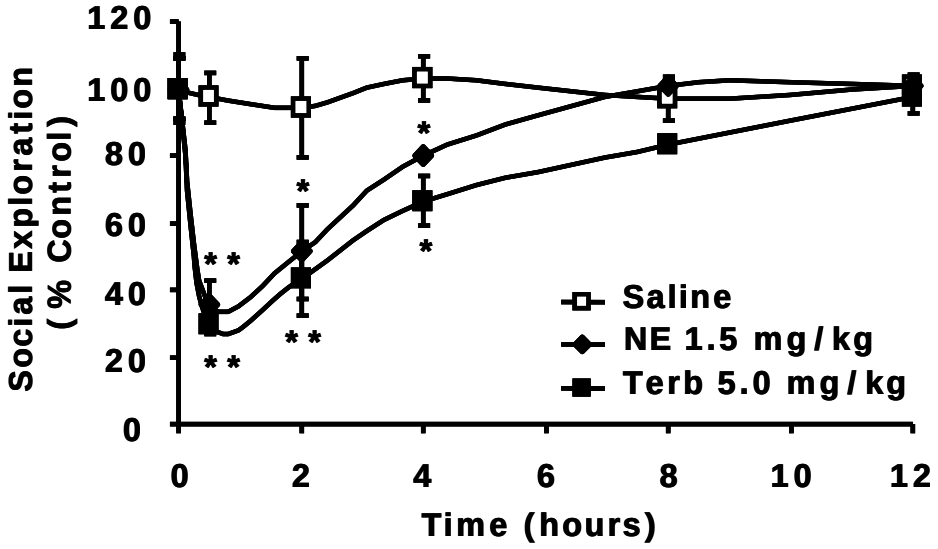


Fig 3.2. Catecholamines cause social withdrawal. (A) After a 12h fast, C57BL/6J mice were administered either insulin (Insulin) or saline control (Saline) at 0.8 units/kg insulin IP as indicated. Plasma catecholamines were measured by HPLC at 0, 45 and 120 min post insulin injection. Results are expressed as mean ± SEM; n = 3, *P <0.01, **P <0.001 Insulin vs. Saline. (B) Mice were administered NE (IP) at the concentrations indicated. Social exploration was measured at 0, 0.5, 2, 4, 8 and 12h after NE delivery. Results are expressed as percentages of the baseline measurement, means ± SEM; n=4. *P<0.01, **P < 0.0001, NE vs. Saline. (C) Mice were administered Epi (IP) at the concentrations indicated. Social exploration was measured at 0, 0.5, 2, 4, 8 and 12h after Epi delivery. Results are expressed as percentages of the baseline measurement, means ± SEM; n=4, *P<0.01, **P < 0.0001, Epi vs. Saline.

Figure 3.3

(A)


Social Exploration (% Control)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
Time (hours)
Saline
NE 1.5 mg/kg
Terb 5.0 mg/kg
* *
* *
*
*
*

(B)

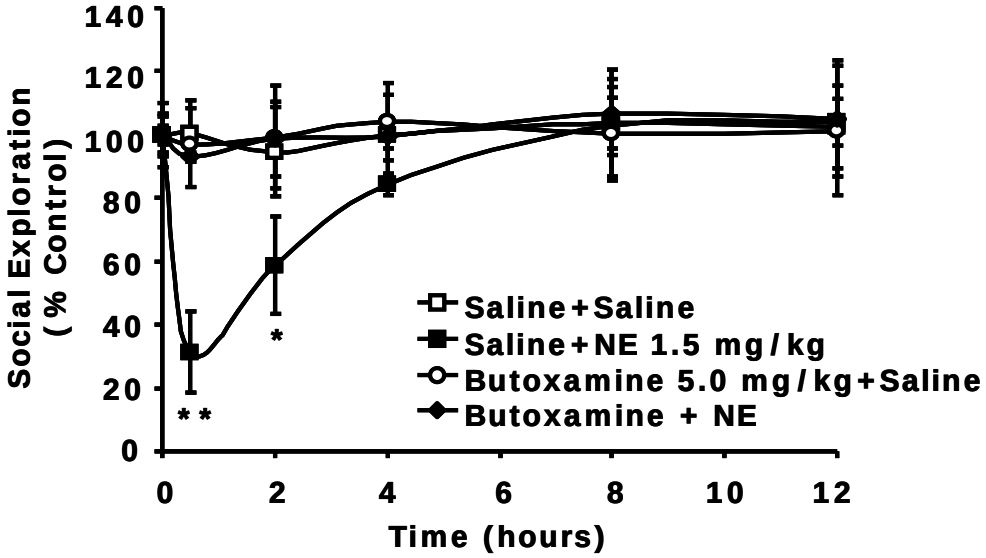
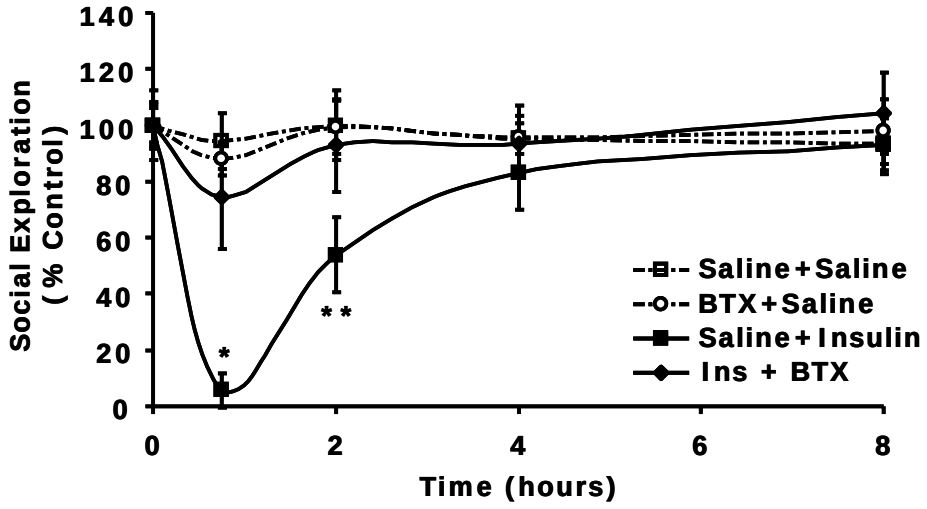

Social Exploration (% Control)
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
Time (hours)
Saline+Saline
Saline+NE 1.5 mg/kg
Butoxamine 5.0 mg/kg+Saline
Butoxamine + NE
* *
*

Fig. 3.3, continued

Fig 3.3. β-2 adrenergic receptor stimulation causes social withdrawal which β-2 adrenergic receptor antagonism prevents. (A) C57BL/6J mice were IP administered NE, terbutaline (Terb) or saline control (Saline) at the concentrations indicated. Social exploration was measured at 0, 0.5, 2, 4, 8 and 12h after injection. Results are expressed as percentages of the baseline measurement, means ± SEM; n=3, *P<0.01, **P < 0.0001, NE or Terb vs. Saline. (B) Mice were IP administered NE, butoxamine (Butoxamine) or Saline at the concentrations indicated. Social exploration was measured at 0, 0.5, 2, 4, 8 and 12h after injection. Results are expressed as percentages of the baseline measurement, means ± SEM; n=3, *P < 0.05, **P < 0.0001, NE vs. butoxamine + NE.

Figure 3.4

(A)


Social Exploration (% Control)
140
120
100
80
60
40
20
0
0
2
4
6
8
Time (hours)
Saline+Saline
BTX+Saline
Saline+Insulin
Ins + BTX
*
* *

(B)

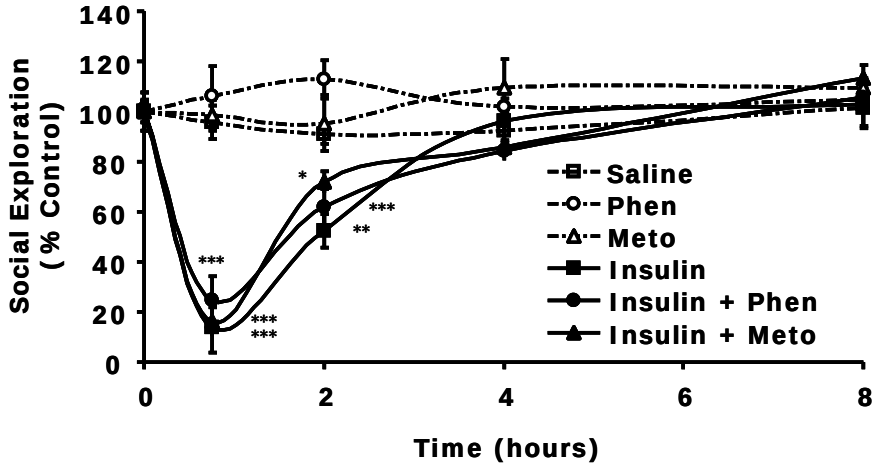

Social Exploration (% Control)
140
120
100
80
60
40
20
0
0
2
4
6
8
Time (hours)
Saline
Phen
Meto
Insulin
Insulin + Phen
Insulin + Meto
***
*
**
***
***
***

Fig. 3.4, continued

Fig. 3.4. Butoxamine blocks insulin-induced social withdrawal. (A) After a 12h fast, C57BL/6J mice were pretreated with or without butoxamine (BTX) (5 mg/kg, IP, pretreatment for 30 min) as indicated. Mice were then administered insulin (Ins) (0.8 units/kg, IP or saline control (Saline) IP as indicated. Social exploration was measured at 0, 0.75, 2, 4 and 8h after insulin delivery. Unrestricted access to food was provided after the 0.75 time point. Results are expressed as percentages of the baseline measurement, means ± SEM; n=3, *P < 0.0001, **P = 0.0007 Insulin vs Insulin + butoxamine. (B) Like in A, C57BL/6J mice were pretreated with either phentolamine (Phen) (1 mg/kg, IP) or metoprolol (Meto) (10 mg/kg, IP), as indicated. Mice were then administered insulin (0.8 units/kg) or saline control IP. Social exploration was measured as in A. Results are expressed as percentages of the baseline measurement means ± SEM; n=4-6, *p=0.0126 **p=0.0018 ***p<0.0001 Saline vs. Insulin +/- Phen or Meto.

CHAPTER 4: HYPOGLYCEMIA/HYPERINSULINEMIA CAUSE DEPRESSIVE-LIKE BEHAVIORS IN MICE

4.1 Abstract

In humans, hypoglycemia is associated with a variety of mood changes including a reduction in hedonic tone and energetic arousal, and an increase in tense arousal. While these clinical symptoms are well characterized, their underlying mechanism is not. C57BL/6J mice were administered 0.8 units/kg insulin that generated a blood glucose nadir of 50 ± 2 mg/dl 0.75h after injection. At 4h post-insulin administration, blood glucose had returned to normal (148 ± 10 mg/dl). Saccharin preference testing 24h post-hypoglycemia showed that insulin-receiving mice had saccharin aversion (62 % vs 91 % of total fluid consumption) that took 48h to resolve. In addition, insulin-treated mice had increased immobility in the forced swim test that took 48h to rectify. Activity, burrowing, elevated zero maze and novel object recognition were not impacted 24h post hypoglycemia. Insulin at 0.8 units/kg increased plasma epinephrine (814 ± 254 pg/ml vs. 350 ± 40 pg/ml) and norepinephrine (541 ± 155 pg/ml vs. 265 ± 28 pg/ml) 24 h-post insulin treatment. Importantly, blocking adrenergic receptors or treatment with anti-depressants ablated the behaviors of insulin-induced saccharin aversion and increased immobility in the forced swim test. Taken together, these data indicate that anhedonia

and depressive-like behavior are induced by hypoglycemia, and these behaviors are dependent on catecholamines in an adrenergic receptor-mediated manner.

4.2 Introduction

Hypoglycemia is the most common acute side effect in insulin-treated subjects with type 1 diabetes (T1D), both in children (Becker and Ryan, 2000) and adults (Frier, 2008), and it is one of the biggest hurdles in the improvement of glycemic control (Cryer, 1994; Cryer, 2002; Cryer, 2008; Havlin and Cryer, 1988; Nery, 2008). Many research studies have focused on the cognitive impact of hypoglycemia, but less research has investigated the psychophysiological bases of such states, including depression. Recent studies by Gold et al. (1995, 1997) have reported biological changes in mood during insulin-induced hypoglycemia. In both non-diabetic and diabetic subjects, Gold et al. (1995) found that acute hypoglycemia caused negative mood states including tense-tiredness, a reduction in hedonic tone (less happy), an increase in tense arousal (more tense) and a decrease in energetic arousal (less energetic). A study by McCrimmon et al. (1995) of 16 healthy individuals exposed to insulin-induced hypoglycemia has confirmed and extended the findings of Gold et al (1995). The healthy individuals presented increased anger and became more pessimistic about their life problems in addition to a

reduction in hedonic tone and an increase in tense arousal. Moreover, in a study of 1200 hypoglycemic patients, Gyland (1953) found that 3 out of 4 hypoglycemic patients showed symptoms of depression and 2 out of 3 patients had worrying and anxiety. More recently, reduced glucose metabolism in the brains of patients with depression has been verified by positron emission tomography (PET) scans (Baxter et al., 1989; Kumar et al., 1993).

Symptoms of hypoglycemia are classified into two categories: autonomic and neuroglycopenic. Autonomic symptoms are the result of the impaired perception of physiologic changes caused by autonomic nervous (sympatho-adrenal) system activation in response to hypoglycemia (Deary et al., 1993; Hepburn et al., 1991; Towler et al., 1993). The stimulation of autonomic centers in the hypothalamus triggers a peripheral autonomic discharge, where catecholamine-mediated adrenergic symptoms comprise sweating, anxiety, tremulousness, palpitations and paresthesias (Diedrich et al., 2002; Field, 1989; Hoffman, 2007). Neuroglycopenic symptoms occur during hypoglycemia as a direct result of a lack of glucose requirement for brain function and are characterized by symptoms such as difficulty in thinking and speech, weakness, tiredness and sleepiness (Diedrich et al., 2002; Gold et al., 1995).

The counter-regulatory hormonal changes in response to hypoglycemia are well

defined. In the normal physiological condition, hypoglycemia counter-regulation includes three responses: 1) secretion of norepinephrine from sympathetic postganglionic nerve terminals, epinephrine from the adrenal medullae and cortisol/corticosterone from the adrenal cortex; 2) secretion of growth hormone by the anterior pituitary gland; and 3) secretion of glucagon and pancreatic polypeptide from the pancreas (Diedrich et al., 2002; Musselman et al., 2003). The acute metabolic effects of the harmonized neuroendocrine response involve increased glucose production and restrained glucose utilization through proteolysis, lipolysis, glycogenolysis and gluconeogenesis (Diedrich et al., 2002; Musselman et al., 2003). Studies of the relationship between depression and glucose metabolism have shown that patients with major depression demonstrate insulin resistance during insulin tolerance tests and intravenous or oral glucose tolerance tests. An important report from adrenalectomized subjects suggests that epinephrine has a pivotal role in changes in hedonic tone, tense arousal and energetic arousal (Hepburn et al., 1996). While control subjects showed a reduction in happiness and an increase in tension after acute hypoglycemia, adrenalectomized patients did not present either symptom (Hepburn et al., 1996). This indicates that Epi release from adrenal glands is a required component of tense arousal induced by hypoglycemia.

Based on the relationship between depression and hypoglycemia, it is not

surprising that both have symptoms in common: nervousness, irritability, exhaustion, drowsiness, insomnia, constant worrying, mental confusion, rapid pulse, internal trembling, forgetfulness, headache and unprovoked anxieties. While these clinical symptoms are well characterized in humans, their underlying mechanism and experimental models in mice are not well established. Here we investigated the mechanism by which insulin-induced hypoglycemia causes depressive-like behavior and anhedonia in mice.

4.3 Materials and methods

Materials

All reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except for Humalin R (insulin), which was purchased from Eli Lilly (Indianapolis, IN).

Animals

All animal care and use was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council). C57BL/6J mice were bred in-house from mice purchased from The Jackson Laboratory. Mice were group housed (4-8) in standard shoebox cages (17.15 x 28 cm) in a temperature (23°C) and

humidity (45–55%) controlled environment with a 12-h/12-h dark-light cycle (0800h to 2000 h). Mice were fed pelleted food (NIH 5K52; LabDiet; Purina Mills) and water *ad libitum*. Male 8- to 12-wk-old animals were used for all experiments. Animals were administered Epi and NE at the indicated concentrations via IP injection. Insulin was administered IP at 0.8 units/kg/mouse or ICV at 0.2 units/mouse/2 µl injection after 12 hours of fasting when indicated. Phentolamine (1mg/kg/mouse), metoprolol (10mg/kg/mouse) or butoxamine (5mg/kg/mouse) were administered IP twice, 30 min prior to insulin injection and 30 min prior to the forced swim test (FST). Antidepressants fluoxetine (10 mg/kg/mouse) or desipramine (5mg/kg/mouse) were also administered IP 30 min prior to FST.

Locomotor Activity Test (LAT)

To estimate locomotor activity, mice were kept in their home cage and video recorded during 5 min tests using a camera mounted approximately 65.0 cm directly above the center of the cage floor. On the video records, cages were divided into four identical rectangles and a trained observer who was blind to experimental treatments determined the frequency of line crossing. A mouse was considered to have crossed a line only if its fore and hind limbs entered a new rectangle.

Forced Swim Test (FST)

The FST measures depressive-like behavior and was conducted as described by Porsolt (2000). Briefly, each mouse was placed individually in a cylinder (diameter: 16 cm; height: 31 cm) containing 15 cm of water maintained at 25 ± 1 °C. The water was changed and the cylinders were cleaned thoroughly between testing sessions. Mice were tested for 6 min and then returned to their home cage. The duration of immobility, swimming and climbing was evaluated during the last 5 min of the test.

Tail Suspension Test (TST)

The TST was carried out as previously described (Steru et al., 1985). Briefly, an adhesive tape was fixed to the mouse tail (distance from the tip of the tail = 2 cm) and hooked to a horizontal ring stand bar placed 30 cm above the floor. The test was conducted for a period of 10 min in a visually isolated area. The apparatus was cleaned thoroughly after each mouse. Mice demonstrated several escape attempts interspersed with immobility periods during which they hung passively and completely motionless. Each mouse was repeatedly tested 24 h, 48h and 72h after treatment. It has been previously shown that exposure to the TST can be repeated without causing any significant habituation (El Yacoubi et al., 2003).

Saccharin Preference Test (SPT)

The saccharin preference test measures anhedonia, pleasure avoidance, a fundamental characteristic of depression (Willner, 1997). Individual mice were housed in standard cages fitted with adapted wire tops to allow access to two fluids: water or 0.4% sodium saccharin solution. Prior to experimental treatment, mice were exposed to two bottles: one for water and another for 0.4% sodium saccharin solution, in order to acclimate them to the presence of two fluid sources in the cage. In order to avoid any place preference, the relative location (left or right) of the saccharin tube was changed whenever fluid intake was measured. Fluid consumption was measured by volume of fluids before and/or after each test session.

Novel object recognition test

To investigate a function of hippocampal-dependent working memory, mice were familiarized with a pair of identical objects which were located near opposite corners of the same end of a large shoebox cage. During the acclimation period, the mouse was exposed to the object for 1-6 h. Before and after the object was placed in the adult's cage, it was thoroughly cleaned with 70% ethanol and dried to sanitize it and ensure the absence of olfactory cues on the object. On the test day, 24h after the end of

the acclimation period, mice were re-exposed to the two familiar objects from the acclimation period (sample phase). Both familiar objects were placed near the opposite corners of the same end of a large shoebox mouse cage (fresh and clean cage). The mouse was then placed in the center of the cage, opposite from the objects. The mouse was allowed to freely explore the cage and the familiar objects for five minutes. The mouse was then removed from the shoebox cage and returned to its home cage, and the objects were removed, cleaned with 70% ethanol and dried again. The mice were then exposed to insulin. Immediately following treatment, mice were placed in a cage just as in the familiar object period, except one of the two familiar objects was replaced with a novel object (recall phase). The mouse was allowed to explore each of the objects for a 5 minute period, after which the mouse was removed and replaced into its home cage. The recall phase was repeated at time points up to 24h post-treatment. To test for the effects of insulin on memory formation, the mice were first exposed to hypoxia, then given a set amount of time to recover (up to 24 hours), then introduced to the novel object test.

Elevated zero maze

Elevated zero maze consisted of a circular platform (6-cm width with a 40-cm inner diameter) that was equally divided into four quadrants. Two quadrants on opposite

sides of the platform were enclosed by walls (14 cm high); the other two quadrants were open and bordered by a lip (0.3 cm high). The maze was elevated 72 cm above the floor. The mouse was placed just inside a closed arm with all four paws inside and its nose pointing inside the closed arm. The mouse was observed and video-recorded for 5 minutes then replaced in its home cage. The maze was cleaned with 75% ethanol between runs. Mice were monitored in real-time while they were being video-recorded.

Burrowing test

Burrowing tubes were constructed from a 2 inch diameter polyvinyl chloride (PVC) conduit. Sections of pipe were cut to 200mm in length, and included an end cap on one end. At the open end of the tube, two small bolts were inserted to prop up the tube, and fastened in place with a nut, with the threading coated in nylon, to prevent loosening. The end product was an open ended (one end only) tube approximately 200 mm in length, with the open end propped up approximately 60 mm. For acclimation, baseline and experimental treatment evaluation of burrowing activity, the mice received their respective treatment (no treatment for acclimation or baseline), and the burrowing tube was placed in the corner of a large conventional mouse cage. Pelleted food (100-200 g) was placed on the floor of the burrowing tube. During the first 6 hours after initial

placement of the burrowing tube in the cage, the amount of food displaced by the mouse's burrowing activity was measured and refilled (to avoid a ceiling effect) at 1h intervals. At the end of the 12 hour testing period, the amount of food displaced from the tube was measured and the tube and its contents were removed from the home cage.

Blood Glucose

Blood was collected from the tail blood as we described previously (Hartman et al., 2004). Briefly, blood glucose levels were measured using a One Touch Ultra glucometer (Johnson & Johnson, Milpitas, CA) per the manufacturer's instructions. In brief, mice were placed in a very shallow shoebox sized container (17.15 x 28 x 4 cm) such that the tail was exposed. The tip of the tail was then secured against the top of the container, snipped and blood drawn. Blood glucose was measured on the same mice utilized in the behavioral experiments.

Plasma Catecholamine Analysis

After the indicated treatments, mice were anesthetized with sodium ketamine hydrochloride:xylazine hydrochloride (80 mg/ml:12 mg/ml, ketamine:xylazine) at 1.5 ml/kg body weight and blood removed from the left ventricle. Blood was collected into

chilled heparinized centrifuge tubes and spun at 9300 x G for 8 min. Plasma was aspirated and stored at -80^{o} C. Catecholamines were determined from plasma by reverse-phase high performance liquid chromatography (HPLC). Solid phase extraction was with aluminum oxide (Bioanalytical Systems, West Lafayette, IN) and elution was in 0.2 N perchloric acid. Dihydroxybenzylamine was used as an internal standard to determine extraction efficiency. Electrochemical detection (ESA, Chelmsford, MA) utilized a 150 x 2 mm C_{18} (3 μm) Hypersil column (Keystone Scientific, Bellfonte, PA) fitted with a 2 mm C_{18} (3 μm) Hypersil javelin guard column (Keystone Scientific). Mobile phase (pH = 3.0) was 75 mM NaH_2PO_4, 1.7 mM 1-ocatanesulfonic acid, 25 μM Na_2EDTA, 7% (vol/vol) acetonitrile and 0.1% (vol/vol) triethylamine. The interassay coefficient of variation was less than 3%.

Statistical Analysis

Data are presented as mean ± SEM and were analyzed by one- or two-way ANOVA depending on the experimental design with repeated measurements in the time factor as applicable. Post hoc comparisons of individual group means were carried out with the Tukey test. Statistical significance was denoted at $P < 0.05$.

4.4 Results

Blood glucose changes

Table 4.1 demonstrates that when C57BL/6J mice were food deprived for 12 h, blood glucose ranged from 99 ± 4 to 103 ± 3 mg/dl. When mice were injected IP with insulin, blood glucose fell. Blood glucose 0.75h after 0.8 units/kg of insulin was 50 ± 2 mg/dl ($p < 0.001$) compared to control (105 ± 4 mg/dl). Food was made accessible to the mice 0.75h post-injection. At 2h post-insulin injection and 1.25h after a return to unrestricted food access, blood glucose ranged from 172 ± 5 to 166 ± 4 mg/dl in control and insulin-treated animals. Blood glucose normalized to 150 ± 10 and 148 ± 10 mg/dl in control and insulin-treated animals by 4h post- injection, and 3.25h after a return to unrestricted food access.

Insulin-induced hypoglycemia is associated with depressive-like behavior and anhedonia.

Fig. 4.1 shows the impact of insulin administration on mobility. The total number of crossings (**Fig. 4.1A**) 24h after 0.8 units/kg of insulin (80 ± 7) was not significantly different from that of control mice (78 ± 9) and the total distance moved (**Fig. 4.1B**) 24h after 0.8 units/kg of insulin (1533 ± 60 cm) was also not significantly different from that

of saline-treated mice (1677 ± 141 cm).

To test whether acute insulin induces depressive-like behavior, the FST and TST were conducted. In animal models, FST and TST are the most commonly used tools for screening depressive-like behaviors (Dantzer et al., 2008; Dantzer, 2009; Petit-Demouliere et al., 2005; Steru et al., 1985). As shown in **Fig. 4.2A**, immobility during FST was increased 24h after acute IP insulin treatment, 138 ± 9 seconds compared to 99 ± 6 seconds ($p=0.004$). Recovery from insulin-induced increased immobility in FST took 48 h. However, acute insulin treatment did not significantly affect behavior in the TST.

Finally, a saccharin preference test was conducted to assess hedonic tone. Depressed mice are reported to show a decreased preference for sweet solutions (Dantzer et al., 2008; Dantzer, 2009). As shown **Fig. 4.3**, insulin-treated mice showed a decrease in saccharin preference within 24h after insulin injection. These findings indicate that insulin-induced hypoglycemia is associated with depressive-like behavior and anhedonia, but not mobility.

To investigate a function of hippocampal-dependent working memory (Sutcliffe et al., 2007), anxiety (Shepherd et al., 1994) and behavioral dysfunction (Deacon, 2006), a novel object recognition test **(Fig. 4.9A)**, elevated zero maze **(Fig. 4.9B)** and a burrowing test **(Fig. 4.9C)** were conducted. However, behaviors in these tests were not

significantly affected by acute insulin treatment.

Increased immobility in FST is improved by antidepressant treatment.

Most antidepressant medications work by increasing the levels of one or more of the monoamines such as dopamine, serotonin or norepinephrine in the synaptic cleft between neurons. To ensure that increased immobility in the FST is directly associated with depressive-like behavior (Dantzer et al., 2008), antidepressants were given with or without insulin 0.5h prior to the FST. Two different antidepressants were used: a norepinephrine and serotonin reuptake inhibitor (desipramine), and a selective serotonin reuptake inhibitor (fluoxetine, = Prozac). As shown in **Fig. 4.4**, increased immobility in FST was improved by both antidepressant treatments, indicating insulin-induced hypoglycemia is associated with depressive-like behavior.

Hypoglycemia increased plasma catecholamines and corticosterone.

Fig. 4.5A demonstrates that 0.8 units/kg insulin IP induced a marked elevation in plasma Epi and NE. At 0.75h after insulin, Epi was increased compared to control, 921 ± 256 pg/ml vs 350 ± 40 pg/ml ($p = 0.044$). At 72h after insulin, Epi returned to near control levels, 738 ± 343 pg/ml vs 350 ± 40 pg/ml ($p = 0.242$). After insulin (0.75 h), NE

increased to 787 ± 311 pg/ml vs 265 ± 28 pg/ml (p = 0.102) and was elevated at 24h post-insulin, 541 ± 155 pg/ml vs 265 ± 28 pg/ml (p = 0.08).

Fig. 4.5B indicates that 0.8 units/kg insulin IP induced a marked elevation in plasma corticosterone. At 0.75h after insulin, corticosterone showed a non-significant increase (p = 0.87) compared to control, 163 ± 38 ng/ml vs 80 ± 14 ng/ml. At 24h after insulin, plasma corticosterone levels were significantly higher, 325 ± 23 ng/ml vs 119 ± 32 ng/ml (p = 0.02).

Exogenous catecholamines cause depressive-like behavior.

To establish if increased catecholamines mediate behavioral changes, we treated mice with Epi and NE and FST was measured. **Fig. 4.5C** shows the impact of insulin administration on mobility. Epi and NE did not influence the total distance moved at 24h post-IP injection, as measured by Noldus EthoVision. Two hour data used for a negative control indicated that Epi and NE induced loss of activity, as shown in a previous study (Park et al., 2008). **Fig. 4.5D** shows that when Epi was administered IP at 1.5 mg/kg, immobility during FST was significantly increased 24h after injection (108 ± 13 vs. control 71 ± 5, p = 0.0221). FST immobility was also increased at 24h when NE was administered IP at 1.5 mg/kg (127 ± 13 vs. 71 ± 5, p=0.0034) and rectified at 48h (p=0.7).

Taken together, these findings indicate that catecholamines increase immobility in FST.

Does β-2 adrenergic receptor antagonism prevent insulin-induced depressive-like behavior?

Decreased β-2 AR densities seem to be associated with hypoglycemia incidence (blood glucose < 50 mg/dl) and symptomatic hypoglycemia unawareness (Schwab et al., 1993; Schwab et al., 2004), and expression of β-2 AR mRNA appears inversely tied to life stress (Miller and Chen, 2006). To determine if catecholamine-dependent depressive-like behavior is mediated by the AR, α, β-1 and β-2 antagonism were performed using Phentolamine (1 mg/kg, pan-α blocker), Metoprolol (10 mg/kg, β-1 blocker) or butoxamine (5.0 mg/kg, β-2 blocker). Agonists were pretreated 30 min prior to insulin (0.8 units/kg, IP), Epi or NE injection, and 30 min prior to FST test. As shown in **Fig. 4.6**, Insulin-induced depressive-like behavior was blocked by IP administration of the α and β antagonists.

4.5 Discussion

We have previously shown that hypoglycemia/hyperinsulinemia induces social withdrawal and reduces mouse movement that peaks 0.5 to 2h later and gradually wanes.

We also reported that a single dose of insulin was significant enough to increase plasma norepinephrine and epinephrine levels, which might be responsible for the social withdrawal observed in insulin-induced hypoglycemia. This phenomenon could be expected because a low blood glucose level triggers a variety of counter-regulation components including catecholamines, glucagon, growth hormone and cortisol. In addition, the insulin-induced social withdrawal was reversed by β-2 adrenergic receptor blockade (Park et al., 2008). While these data show the early responses to hypoglycemia, the current study focused on the later responses.

Dantzer et al. (2008) reported that peripheral administration of lipopolysaccharide (LPS) induced sickness behavior that peaked between 2-6h then gradually diminished, and depression-like behavior followed sickness behavior 24h later. Using an animal model of cytokine-induced depression, they reviewed similarities between sickness behaviors and depression-like behaviors (Dantzer et al., 2008). For instance, the decrease in motor activity presented by sick animals is similar to the increased immobility during FST. In the same way, the reduced appetite of sick individuals translates into a decreased intake of rewarding aliments, mimicking depression-associated anhedonia. They also suggested using pharmacological validation by antidepressant treatment to confirm depressive-like behavior. Here we show that insulin induced depressive-like behavior

and anhedonia, as measured by increased immobility during FST (Fig. 4.2A), and decreased preference for a sweet solution (Fig. 4.3) 24 hours post-injection. The increased immobility during FST and decreased intake of sweet solution (behavioral validation) were abolished by antidepressant treatment (pharmacological validation) (Fig. 4.4).

An important question arises when considering the insulin-induced increase in immobility during FST. Is it insulin itself or the insulin-induced glucose deficit that is causing this depressive-like behavior? As shown in Table 4.2 and Fig. 4.7, ICV insulin administration neither decreased blood glucose nor increased immobility during FST. More interestingly, as shown in Table 4.3 and Fig. 4.8, insulin did not increase immobility during FST when mice did not reach a hypoglycemic state. These findings strongly support our contention that depression-like behavior is associated with an insulin-induced glucose deficit, but not with insulin per se.

More direct evidence for catecholamine involvement in mood changes comes from a study of adrenalectomized patients (Hepburn et al., 1996). While normal individuals showed increased plasma adrenalin and tense arousal and a decrease in hedonic tome in response to acute insulin-induced hypoglycemia, adrenalectomized patients (as treatment for Cushing's disease) neither secreted adrenaline nor exhibited

tense arousal and changes in hedonic tone under the same condition (Hepburn et al., 1996). These observations suggest that the marked increase in adrenaline secretion from the adrenal medulla plays a critical role in the generation of changes in tense arousal and hedonic tome (Hepburn et al., 1996). In a study of healthy participants, Gold et al. (Gold et al., 1995) reported mood changes induced during insulin-induced hypoglycemia. The acute hypoglycemia caused negative mood states including a reduction in hedonic tone, an increase in tense arousal and a decrease in energetic arousal. McCrimmon et al. (1995) also reported that 16 healthy individuals exposed to insulin-induced hypoglycemia presented increased anger and became more pessimistic about their life problems in addition to a reduction in hedonic tone and an increase in tense arousal (McCrimmon et al., 1995). Hislop et al. (2008) reported approximately one-third of young adults with T1D experience psychological distress, which is associated with poorer glycaemic control (Hislop et al., 2008). These findings of hypoglycemia-induced negative changes in mood including diminished energetic arousal, decreased happiness and increased anxiety in humans, support our result in mice that insulin-induced hypoglycemia caused depressive-like behavior and anhedonia and suggest that circulating plasma catecholamines may be important in mediating such changes (Gold et al., 1997). Therefore, our experimental model of acute hypoglycemia is a useful method

for studying mood in humans and animals (Gold et al., 1995).

Fig. 4.5D demonstrates that peripheral administration of Epi/NE caused increased immobility during FST. These findings indicate that Epi or NE might be responsible for the depression-like behavior seen with insulin-induced hypoglycemia. In a review of the roles of catecholamines in depression, Potter (1994) and many other findings showed that patients with major depression presented some degree of increased plasma NE levels, indicating increased peripheral sympathetic nervous system activity in depressed individuals (Barnes et al., 1983; de Villiers et al., 1987; Lake et al., 1982; Roy et al., 1985; Roy et al., 1987; Rudorfer et al., 1985; Wyatt et al., 1971). More specifically, enhanced levels of plasma NE were found in patients with depression (de Villiers et al., 1987; Rudorfer et al., 1985), anxiety (Wyatt et al., 1971), major affective disorder (Lake et al., 1982) and/or melancholia (Roy et al., 1985). Fig. 4.6 shows that the α, β 1 and β 2 adrenergic receptor blockade reversed hypoglycemia-associated increases in forced swim immobility without raising blood glucose in response to insulin, indicating that insulin-induced depression-like behavior is not directly mediated by hypoglycemia but by the impact hypoglycemia has on catecholamines via an adrenergic receptor-mediated pathway. There are various studies concerning the effects of α and β adrenergic receptor agonists and/or antagonists on biochemical, neuroendocrine or behavioral system.

Studies using α 2 adrenergic receptor agonists (e.g. clonidine or UK-14304) demonstrated an increased maximum binding in platelets of depressed patients (Garcia-Sevilla et al., 1986; Garcia-Sevilla et al., 1990; Pandey et al., 1989; Piletz et al., 1986), providing evidence for an α 2 hypersensitivity theory of depression (Potter and Manji, 1994). Others reported the opposite, showing α 2 inhibition of platelet adenylate cyclase activity suggestive of subsensitive α 2 adrenergic receptors in depression (Kafka and Paul, 1986; Pandey et al., 1990). In addition, the reduction in β adrenergic receptors and sensitivity of β adrenergic receptor-stimulated adenylate cyclase found in rodent brain after chronic administration of all classes of antidepressant treatment indicate that such alterations may be associated with the therapeutic action of antidepressant drugs (Banerjee et al., 1977; Potter and Manji, 1994; Sulser et al., 1978). Increases in circulating catecholamines and glucocorticoids may play an important role in β adrenergic receptor sensitivity (Werstiuk et al., 1990), possibly via their effects on the stimulatory G protein (Gs) (Malbon, 1989; Potter and Manji, 1994). Since the roles of α and β adrenergic receptors in mood disturbances are still controversial across the literature, additional studies are crucial to further our understanding their roles.

4.6 Figures and Tables

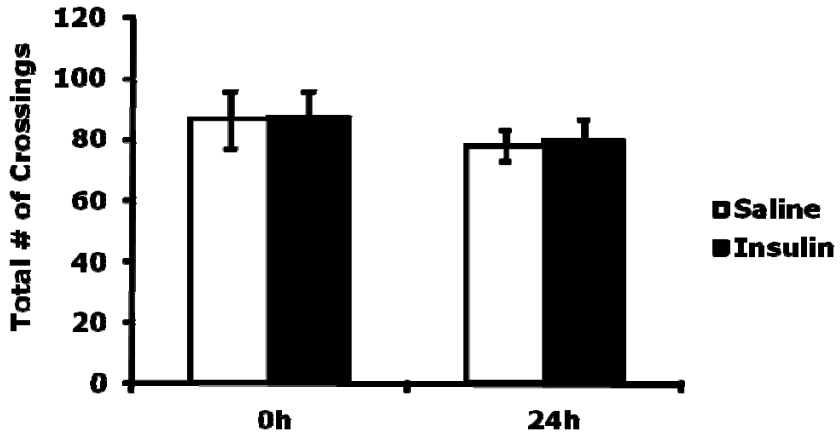
Table 4.1: Blood Glucose (mg/dl) after Insulin IP Injection

	Before fasting (-12h)	0h	0.75h	2h	4h	24h
Saline	152 ± 6	99 ± 4	105 ± 4	172 ± 5	150 ± 10	152 ± 7
Insulin (0.8 units/kg)	149 ± 6	103 ± 3	50 ± 2*	166 ± 4	148 ± 10	145 ± 8

Mean ± SEM, * $p<0.001$, Sal vs. Ins

Figure 4.1

(A) Total number of crossings



(B) Distance moved (cm)

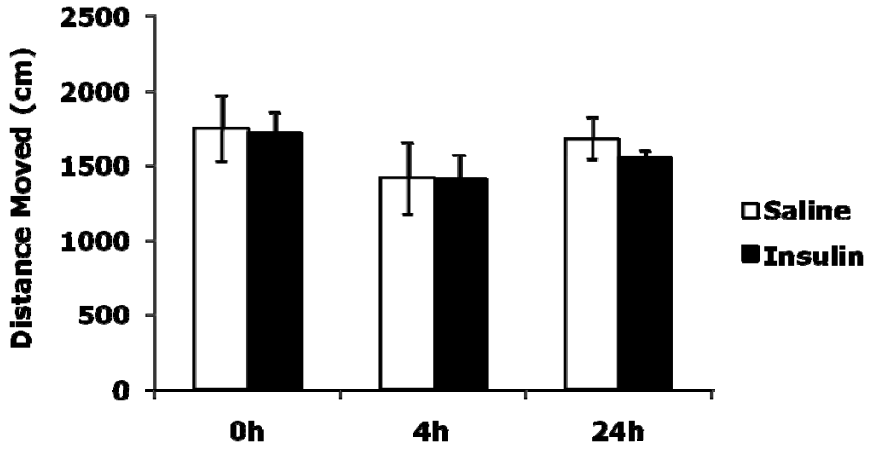
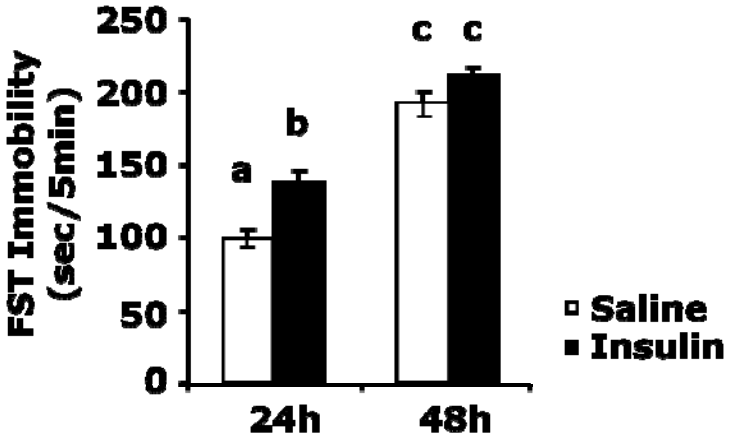


Fig. 4.1, continued

Fig. 4.1. Insulin administration does not impact mobility. After a 12h fast, C57BL/6J mice were administered either insulin or saline control IP as indicated. Locomotor activity was measured at 0, 4 and/or 24h after insulin delivery. Unrestricted access to food was provided within 30 min post-injection. Results are expressed as total line crossings (A) or distance moved as measured by computer program, Noldus (B), means ± SEM; n=4-8.

Figure 4.2

(A) Forced swim test immobility (seconds/5min)



(B) Tail suspension test immobility (seconds/10 min)

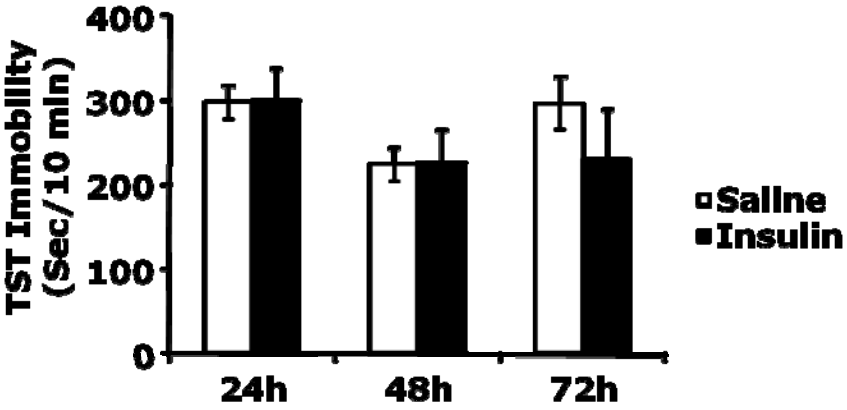


Fig. 4.2, continued

Fig. 4.2. Insulin administration increases FST immobility, but not TST immobility. After a 12h fast, C57BL/6J mice were administered either insulin or saline control IP as indicated. Forced swim test and tail suspension test were measured at 24, 48 and/or 72h after insulin delivery. Unrestricted access to food was provided within 45 minutes post-injection. Results are expressed as immobility in the FST (A) or that of TST (B), means ± SEM; n=14-15 (A) or n=4-8 (B).

Figure 4.3

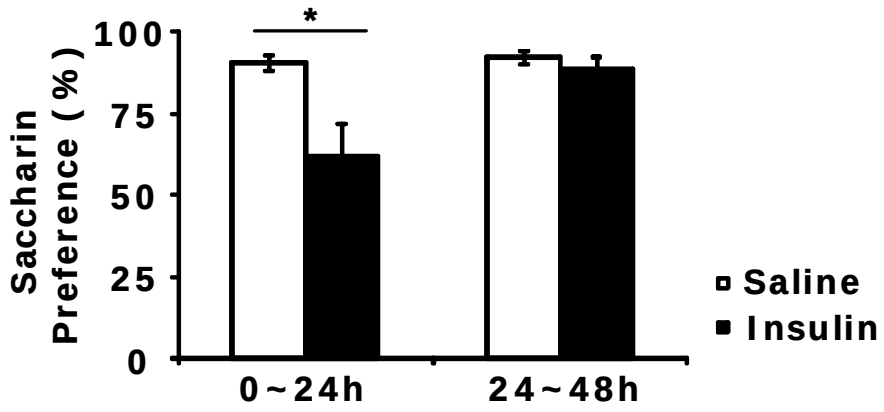


Fig. 4.3. Insulin-treated mice showed a decrease in saccharin preference. After a 12h fast, C57BL/6J mice were administered either insulin or saline control IP as indicated. Saccharin preference test was measured at 24 and 48h post-insulin administration. Unrestricted access to food was provided within 45 minutes post-injection. Results are expressed as Saccharin preference (%) = {Saccharin consumption / (water consumption + Saccharin consumption)} X 100, means ± SEM; n=4; $p<0.05$, Saline vs. Insulin.

Figure 4.4

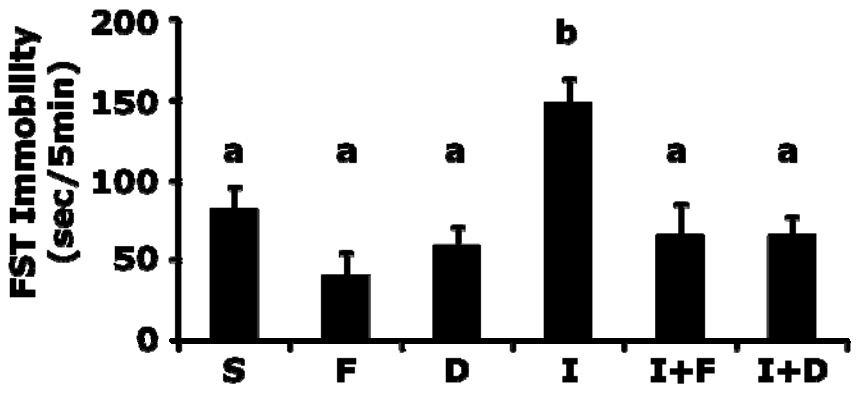


Fig. 4.4. Increased immobility in FST is improved by antidepressant treatment.

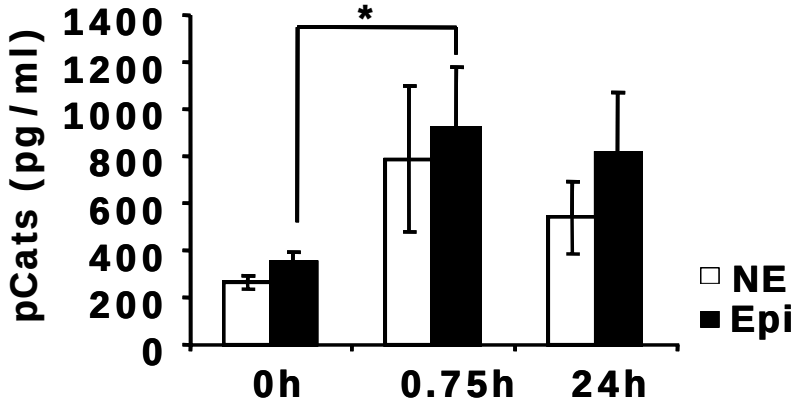
After a 12h fast, C57BL/6J mice were administered either insulin or saline control IP as indicated. 0.5h prior to FST, mice were given saline, fluoxetine or desipramine. Results are expressed as immobility in the FST, means ± SEM; n=9; $p<0.05$.

S, Saline; F, Fluoxetine; D, Desipramine; I, Insulin; I+F, Insulin+Fluoxetine;

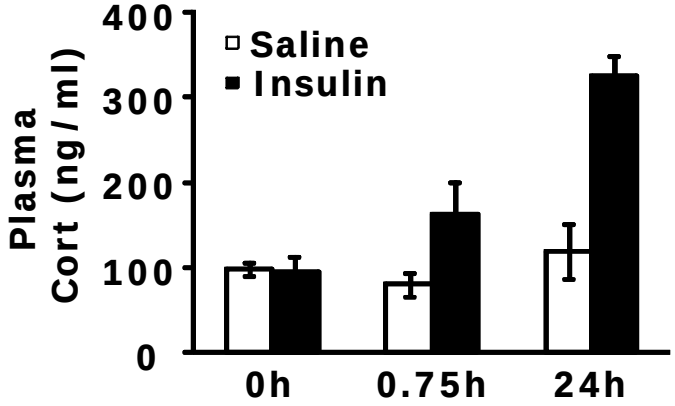
I+D, Insulin+Desipramine

Figure 4.5

(A)



(B)



(C)

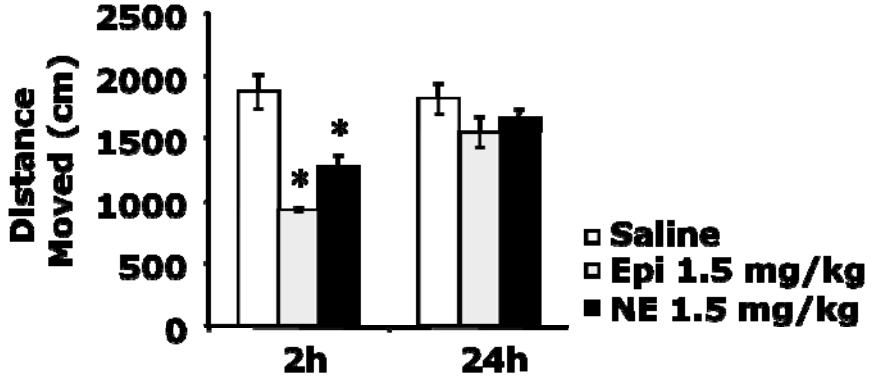


Fig. 4.5, continued

(D)

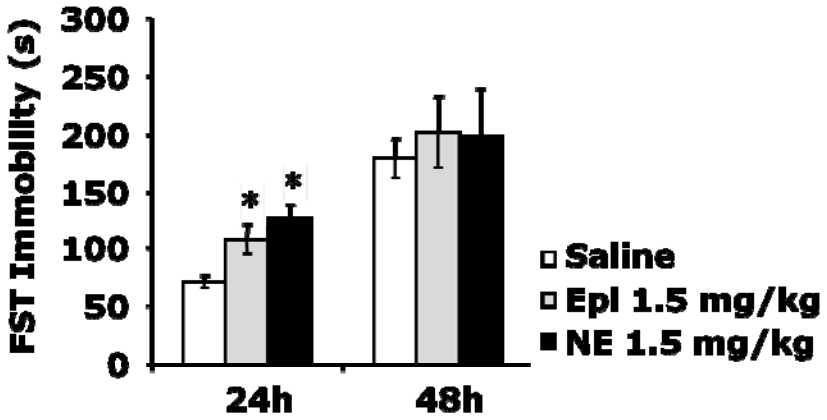


Fig. 4.5. Hypoglycemia causes an increase in plasma catecholamines and corticosterone, and exogenous catecholamines increase immobility in FST. (A) and (B) After a 12h fast, C57BL/6J mice were administered either insulin or saline control at 0.8 units/kg insulin IP as indicated. Plasma catecholamines and corticosterone were measured by HPLC at 0, 0.75 and 24h post-insulin injection. Results are expressed as mean ± SEM; n = 4, *P <0.05, Insulin vs. Saline. (D) Mice were administered Epi (IP) or NE (IP) at the concentrations indicated. FST was measured at 24 and 48h after Epi or NE delivery. means ± SEM; n=4, *P<0.05, Epi or NE vs. Saline at 24 h.

Figure 4.6

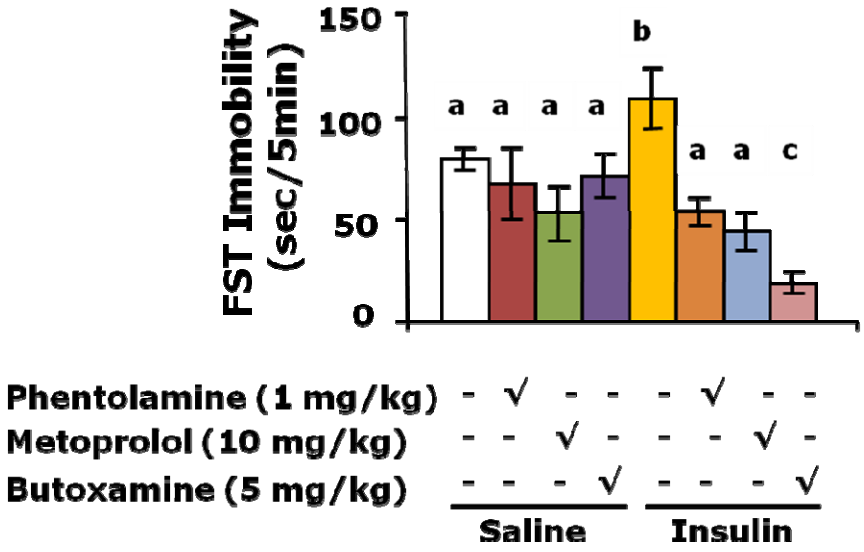


Fig. 4.6. Hypoglycemia-induced increased immobility in FST is improved by AR blockers treatment. (A) After a 12h fast, C57BL/6J mice were pretreated with or without phentolamine (Phen) (1 mg/kg, IP), metoprolol (Meto) (10 mg/kg, IP) or butoxamine (BTX) (5 mg/kg, IP) as indicated. Mice were then administered insulin (Ins) (0.8 units/kg) IP or saline control (Saline) IP as indicated. FST was measured at 24h after insulin delivery. Results are expressed as seconds/5 min of immobility in FST, means ± SEM; n=9; $p<0.05$.

Table 4.2: Blood Glucose (mg/dl) after Insulin ICV Injection

	Before fasting (-12h)	0h	0.75h	2h	4h	24h
Saline, ICV	161 ± 13	114 ± 3	116 ± 8	132 ± 5	131 ± 13	142 ± 13
Insulin, ICV	156 ± 9	108 ± 6	113 ± 9	141 ± 13	136 ± 7	151 ± 14

Mean ± SEM, Insulin ICV at 0.2 units/mouse/2 µl injection.

Figure 4.7

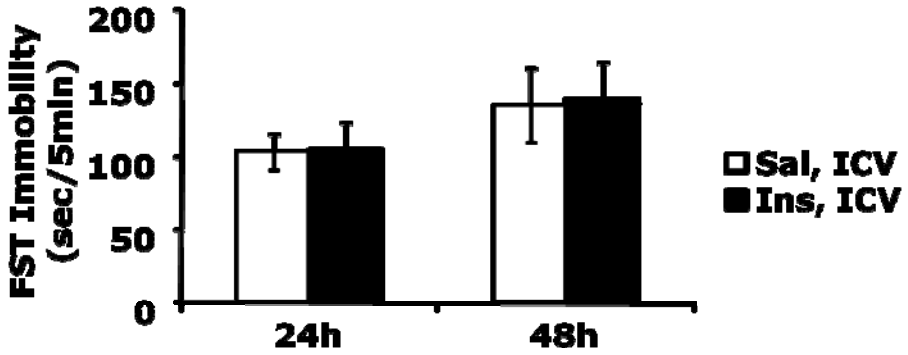


Fig. 4.7. Forced swim test immobility (seconds/5min) post insulin ICV (0.2 units/mouse/2 μl) administration. After a 12h fast, C57BL/6J mice were administered either insulin ICV (0.2 units/mouse/2 μl) or saline ICV as indicated. FST was measured at 24 and 48h after insulin delivery. Unrestricted access to food was provided within 45 minutes post-injection. Results are expressed as immobility in the FST, means ± SEM; n=3-4.

Table 4.3: Blood Glucose (mg/dl) after Insulin IP Injection (without fasting)

	0 min	10 min	20 min	40 min	60 min	80 min
Saline	141 ± 6	152 ± 12	167 ± 7	177 ± 7	172 ± 3	166 ± 5
Insulin	131 ± 15	99 ± 11	101 ± 6	122 ± 1	168 ± 10	172 ± 10

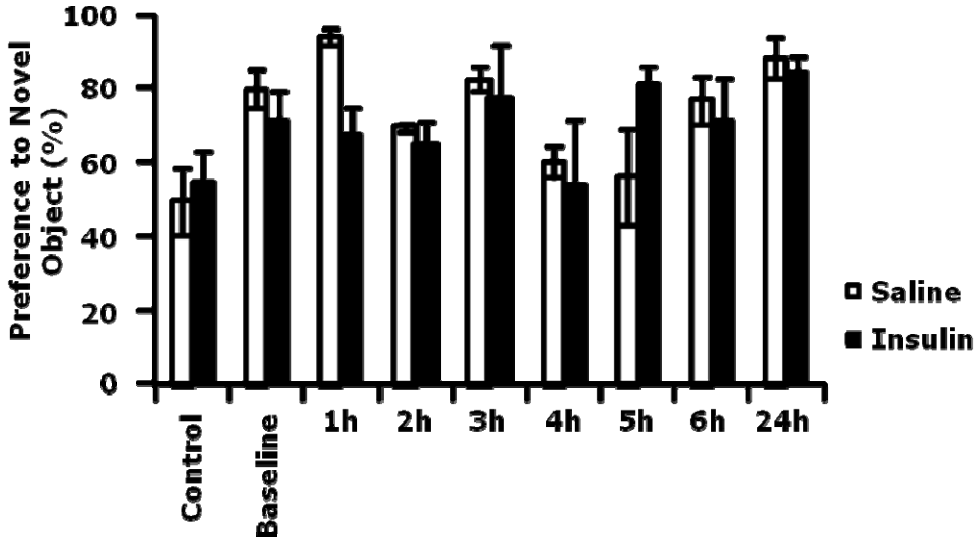
Mean ± SEM

Figure 4.8

Fig. 4.8. Forced swim test immobility (seconds/5 min) post-insulin IP injection (without fasting). C57BL/6J mice were administered either insulin or saline IP as indicated. FST was measured at 24h after insulin delivery. Results are expressed as immobility in the FST, means ± SEM; n=3-4.

Figure 4.9

(A)


Preference to Novel Object (%)
100
80
60
40
20
0
Control
Baseline
1h
2h
3h
4h
5h
6h
24h
Saline
Insulin

(B)

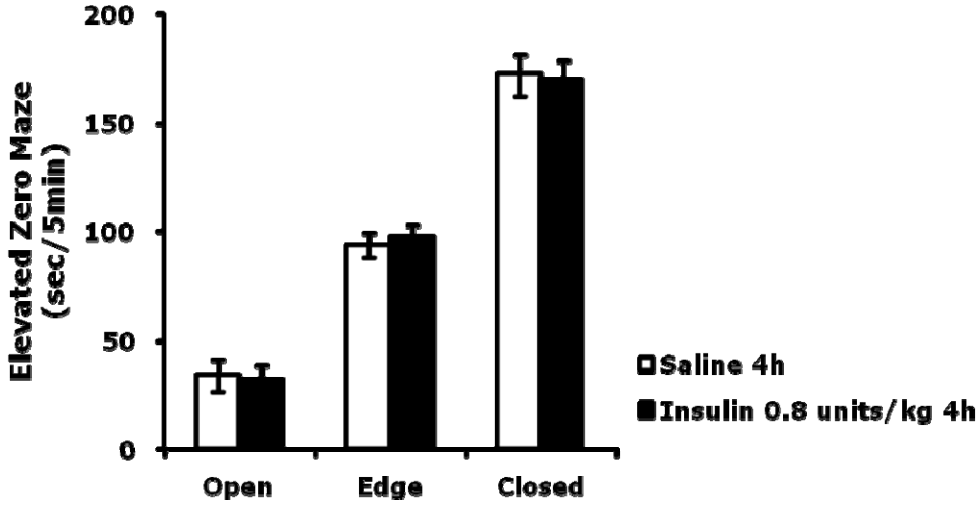

Elevated Zero Maze (sec/5min)
200
150
100
50
0
Open
Edge
Closed
Saline 4h
Insulin 0.8 units/kg 4h

Fig. 4.9, continued

(C)

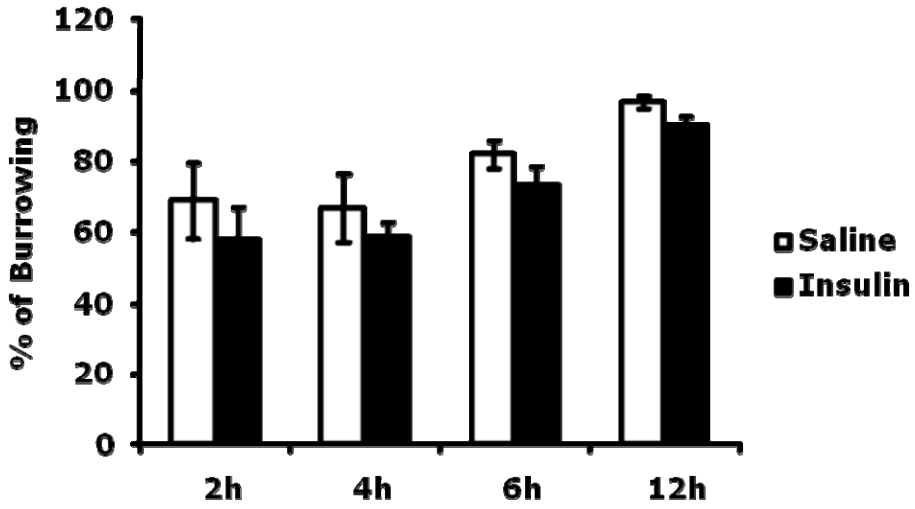
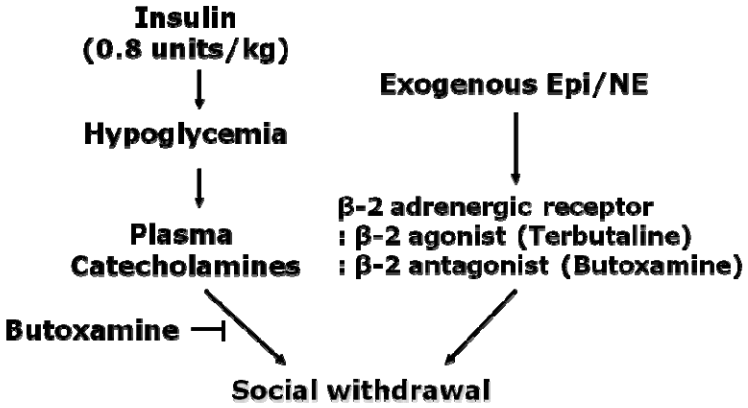


Fig. 4.9. Novel object recognition test, elevated zero maze and burrowing test post-insulin IP injection. C57BL/6J mice were administered either insulin or saline IP as indicated and novel object recognition, elevated zero maze and burrowing test were measured at indicated time points. Means ± SEM; n=4 (A). n=7-9 (B), n=12-14 (C).

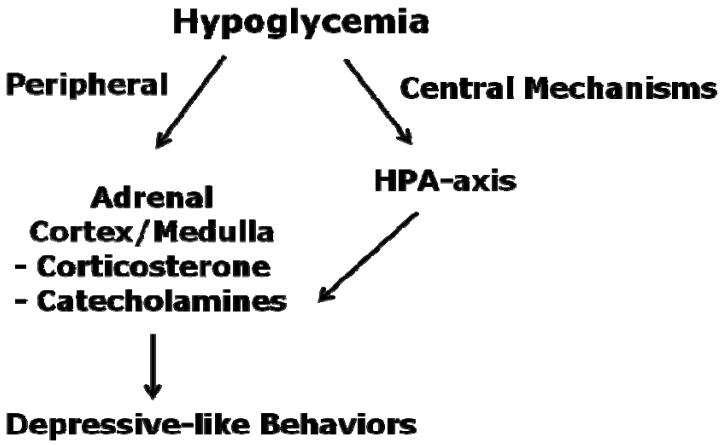
CHAPTER 5: OVERALL SUMMARY AND FUTURE DIRECTIONS

The overall objective of this work was to investigate the possible mechanisms of behavior and mood changes induced by hypoglycemia/hyperinsulinemia. Our first objective was to determine how insulin-induced hypoglycemia causes social withdrawal and if intervention with adrenergic receptor antagonists could speed recovery from social withdrawal. Here we show that hypoglycemia/hyperinsulinemia decreased mouse movement and induced a reduction in social exploration that peaked 0.5-2h later, and then gradually waned. The dose of insulin administered (0.8 units/kg) was significant enough to increase plasma NE and Epi levels, which might be responsible for the social withdrawal observed in insulin-induced hypoglycemia. Importantly, insulin-induced social withdrawal was reversed by β-2 adrenergic receptor blockade (see Summary 1).



Summary 1: Blocking of β-2 Adrenergic Receptor Speeds Recovery from Hypoglycemia-induced Social Withdrawal

The second objective of this dissertation was to investigate how insulin-induced hypoglycemia causes depressive-like behavior and anhedonia. To answer this question, we were interested in determining if an increase in catecholamines mediates depressive-like bahavior in insulin-induced hypoglycemia and if adrenergic receptors are associated with depressive-like behavior and anhedonia. We found that insulin induced saccharin aversion and increased immobility during FST in mice. Importantly, blocking adrenergic receptors or treatment with anti-depressants abolished both these behaviors. Therefore, the data suggest that hypoglycemia-induced anhedonia and depressive-like behavior are dependent on catecholamines in an adrenergic receptor-mediated manner.



Summary 2: Neuroendocrine Mechanisms of Mood Changes induced by Hypoglycemia

As described above, we have shown that insulin-induced social withdrawal and depressive-like behavior are associated with catecholamines and can be reversed by adrenergic receptor blockade. In addition to catecholamines, other studies have shown a relationship between behavioral/mood change and corticotropin-releasing hormone (CRH). CRH is one of the hormones involved in the HPA-axis and is produced by parvocellular neuroendocrine cells of the hypothalamus. CHR is released into the anterior lobe of the pituitary, where it stimulates corticotropes to secrete corticotropin (adrenocorticotropic hormone, ACTH). CRH has been reported to induce behavioral activation including an increase in grooming and freezing behavior, and a decrease in the number of approaches to a food pellet in a rat model (Sutton et al., 1982). Moreover, it has been shown that CRH is anxiogenic in that it suppresses social interaction and potentiates acoustic startle in animal models (Dunn and File, 1987). Holsboer reviewed and reported a potential role for CRH receptor antagonist to treat depression and anxiety (Holsboer, 1999). Most of the behavioral and mood changes induced by CRH were blocked by α-helical CRH_{9-41}, which acts as a CRH receptor antagonist. This α -helical CRH_{9-41} has been reported to suppress the rise of plasma epinephrine after insulin-induced hypoglycemia (Jacobson et al., 2006). Since Epi secretion from the adrenal medulla plays a critical role in the generation of changes in tense arousal and hedonic

tone, further experiments using CRH-KO mice and/or α -helical CRH_{9-41} may show clearer support for our hypothesis that catecholamines and corticosterone play a critical role in behavioral and mood change induced by insulin-induced hypoglycemia.

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psycho-social variables, glycemic control, depression and anxiety. Laeknabladid 94 12:823-829.

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CURRICULUM VITAE

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EDUCATION

2005-2010 Ph.D., University of Illinois at Urbana-Champaign, IL
Ph.D. **Division of Nutritional Sciences**
& Integrative Immunology and Behavior Program
Graduation Date: August, 2010

2002-2004 Master of Science, Pusan National University, Busan, South Korea
M.S. **Department of Molecular Biology**
Graduation Date: February, 2004

1997-2002 Bachelor of Home Economics, Pusan National University, South Korea
B.S. HE Major: **Department of Food Science and Nutrition**
Double Major: **International Studies Program**
Graduation Date: February, 2002

CERTIFICATIONS

- **Certificate in WIPO Summer School on Intellectual Property**
  World Intellectual Property Organization (WIPO), July 2009
- **Certificate in Business Administration**
  College of Business, University of Illinois at Urbana-Champaign, April 2009

RESEARCH AND PROFESSIONAL EXPERIENCES

08/2005-Present Research Assistant, University of Illinois at Urbana-Champaign, IL
- Conducted and developed projects on psychoneuroimmune modulations of hypoxia and hypoglycemia in a mouse model
- Collaborated with faculty to publish papers
- Presented papers and findings at regional and international conferences
- Oversaw the work of other RA's and undergraduates

Supervised by Gregory G. Freund, M.D.

06/2006-Present Director, Korean-American Scientist and Engineers' Association (KSEA), Central Illinois Chapter, Urbana, IL
- Created budget and determined priorities

- Managed fund raising and controlled spending
- Developed and coordinated several workshops (e.g. Young Generation Technical and Leadership Workshop, Math Competition, Career Workshop for Academic Jobs, and Career Workshop for Industry Jobs)
- Built broad networks with both scientists and engineers

Supervised by Christopher Ha, Ph.D. and Andrew Yun, Ph.D.

03/2004-06/2005 Research Specialist, Department of Molecular Biology
Pusan National University, Busan, South Korea

- Coordinated, planned and conducted training programs for lab technicians and students
- Managed projects on necrosis and apoptosis regulation in a cell model

Supervised by Ho Sung Kang, Ph.D.

03/2002-02/2004 Research Assistant and Teaching Assistant in Master's program
Department of Molecular Biology, Pusan National University, Busan, South Korea

- Taught units as a teaching assistant
- Thesis title "Inhibition of Reactive Oxygen Species (ROS) Production and Activation of PKC Switch Glucose Deprivation-induced Cell Death Mode in A549 Human Lung Adenocarcinoma Cells"
- Conducted and managed a project on Health Technology R&D Program supported by a grant from the Ministry of Health & Welfare, South Korea
- Coordinated, planned and conducted a project on National Cancer Control R&D Program supported by a grant of the Ministry of Health & Welfare, South Korea

Supervised by Ho Sung Kang, Ph.D.

03/2001-07/2001 Research Assistant and Undergraduate Student, Department of Food Science and Nutrition, Pusan National University, Busan, South Korea

- Conducted a project on *kimchi*'s potential for controlling obesity and arteriosclerosis using the following methods: solvent fraction of *kimchi*, plasma lipoprotein isolation, extraction of fatty acids, measurement of triglyceride and cholesterol

Supervised by Yeong Ok Song , Ph.D.

PUBLICATIONS

Park MJ, Yoo SW, Choe B, Dantzer R, Freund GG. Hypoglycemia/hyperinsulinemia cause depressive-like behaviors in mice. ***In preparation***

Park MJ, Guest CB, Barnes MB, Martin J, Ahmad U, York JM, Freund GG. Blocking of β-2 adrenergic receptors hastens recovery from hypoglycemia-associated social withdrawal. ***Psychoneuroendocrinology (2008)***, 1411-1418

Park MJ*, Guest CB*, Johnson DR, Freund GG. The implication of proinflammatory cytokines in type 2 diabetes. ***Frontiers in Bioscience (2008),*** 5187-5194 (* contributed equally to this work)

Kim CH, Han SI, Lee SY, Youk HS, Moon JY, Duong HQ, **Park MJ**, Joo YM, Park HG, Kim YJ, Yoo MA, Lim SC, Kang HS. Protein kinase C-ERK1/2 signal pathway switches glucose depletion-induced necrosis to apoptosis by regulating superoxide dismutases and suppressing reactive oxygen species production in A549 lung cancer cells. ***Journal of cellular physiology (2007)***, 371-385

Seo MS, Oh SY, **Park MJ**, Kim SM, Kim MY, Han SI, Park HG, Kang HS. Implication of reactive oxygen species, ERK1/2, and p38MAPK in sodium salicylate-induced heat shock protein 72 expression in C6 glioma cells. ***International journal of molecular medicine (2005)***, 841-849

Oh SY, Kim JH, **Park MJ**, Kim SM, Yoon CS, Joo YM, Park JS, Han SI, Park HG, Kang HS. Induction of heat shock protein 72 in C6 glioma cells by methyl jasmonate through ROS-dependent heat shock factor 1 activation. ***International journal of molecular medicine (2005)***, 833-839

PROFESSIONAL PRESENTATIONS

Min J. Park, Samuel W Yoo, Brian S Choe, Robert Dantzer, and Gregory G Freund, Hypoglycemia causes depressive-like behaviors in mice. Presented at the **Experimental Biology (EB)**, Anaheim, CA, April 28, 2010

Min J. Park, Samuel W Yoo, Brian S Choe, Robert Dantzer, and Gregory G Freund, Hypoglycemia / hyperinsulinemia leads to depressive-like behaviors in mice. Presented at the **Nutrition Symposium**, University of Illinois at Urbana-Champaign, Urbana, IL, April 21, 2010

Min J. Park, Jason C. O'Connor, Desiree N. Lavin, Robert Dantzer, Keith W. Kelley, and Gregory G. Freund. Acute hypoglycemia causes extended anhedonia in mice. Presented at the **Experimental Biology (EB)**, New Orleans, LA, April 22, 2009

Desiree N. Lavin, Christina L. Sherry, **Min J. Park**, Jason C. O'Connor, Robert Dantzer, Keith Kelley, Gregory G. Freund. High fat diet alters expression of catechol-O-

methyl transferase in the brains of mice and decreases motivation to obtain reward. Presented at the **Experimental Biology (EB)**, New Orleans, LA, April 22, 2009

Min J. Park, Jason C.O'Connor, Desiree N. Lavin, Robert Dantzer, Keith W. Kelley, and Gregory G. Freund. Extended anhedonia is induced by acute hypoglycemia in mice. Presented at the **Nutrition Symposium**, University of Illinois at Urbana-Champaign, Urbana, IL, April 14, 2009

Min J. Park, Christopher B. Guest, Meredith B. Barnes, Jonathan Martin, Uzma Ahmad and Gregory G. Freund. Blocking of β-2 adrenergic receptor hastens recovery from hypoglycemia-associated social withdrawal. Presented at the **Psychoneuroimmunology Research Society (PNIRS).** Madison, WI, May 28-31, 2008

Min J. Park, Christopher B. Guest, Meredith B. Barnes, Jonathan Martin, Uzma Ahmad and Gregory G. Freund. Recovery from hypoglycemia-associated social withdrawal is hastened by blocking of β-2 adrenergic receptors. Presented at the **Nutrition Symposium**, University of Illinois at Urbana-Champaign, Urbana, IL, April 2, 2008

Min J. Park, Daniel R. Johnson, Jonathan Martin, and Gregory G. Freund. Norepinephrine-dependent activation of the neuroimmune system is counter-regulated by IL-1RA**.** Presented at the **American Association of Immunologists (AAI).** Miami, FL, May 19-23, 2007

HONORS AND AWARDS

- Abbott Nutrition Scholarship, University of Illinois at Urbana-Champaign, 2009
  Abbott Nutrition supported a scholarship to take a 3-month certification program "Certificate in Business Administration" emphasizing basic accounting concepts, and the fundamentals of organization, leadership, management, reporting, and analysis
- Margin of Excellence Student Travel Award, University of Illinois at Urbana-Champaign, 2009
  Student travel award to international conference on Experimental Biology held in New Orleans, LA
- First Place Poster, Annual Symposium of Division of Nutritional Sciences, University of Illinois at Urbana-Champaign, 2008
  First place award for poster on "Recovery from hypoglycemia-associated social withdrawal is hastened by blocking of β-2 adrenergic receptors"
- Margin of Excellence Student Research Award, University of Illinois at Urbana-Champaign, 2007
  Research award to support a research proposal on "Role of β-2 adrenergic receptor antagonist, butoxamine, in hypoxia-induced social withdrawal"
- Margin of Excellence Student Travel Award, University of Illinois at Urbana-Champaign, 2006

Student travel award to international conference on American Association of Immunologists held in Miami, FL

- Study Abroad Scholarship ($60,000), Korea Science and Engineering Foundation, South Korea, 2005-2007
- Honors Tuition Waiver Scholarship, Pusan National University, South Korea, 2002
- Honors Matriculation and Tuition Waiver Scholarship, Pusan National University, South Korea, 2001
- Honors Matriculation and Tuition Waiver Scholarship, Pusan National University, South Korea, 1997

PROFESSIONAL AFFILIATIONS

- American Society for Nutrition, 2010
- Psychoneuroimmunology Research Society, 2006
- Korean-American Science Engineer's Association (KSEA), 2006-present (Working as a director for Central IL chapter)